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ABSTRACT OF THESIS

MATRIX GEOCHEMISTRY AND *PHYTOPHTHORA* OCCURRENCE ON REFORESTED MINE LANDS IN APPALACHIA

At the Bent Mountain surface mine, Pike County, Kentucky, a study has been ongoing since 2005 to assess the influence of various types of loose-graded mine spoils on water quality and forest establishment. Six research plots consist of two replicates of brown weathered sandstone, gray unweathered sandstone, and mixed brown sandstone, gray sandstone, and shale that were emplaced according to Forestry Reclamation Approach criteria. A series of analyses was initiated in 2007 to examine influence of spoil matrix composition on sulfate and carbonate geochemistry of infiltrated waters, as well as to investigate the occurrence of *Phytophthora*, a group of exotic forest pathogens that cause dieback and may affect success of founder species of hybrid American Chestnut (*Castanea dentata* Marsh.) Borkh.), on the surface of mine spoils and in infiltrated waters.

To identify the constituent responsible for elevated sulfate concentrations, as well as determine the role of alkalinity in spoil waters, PHREEQC geochemical modeling was used to analyze aqueous speciation of sulfates and carbonates with respect to time and tailing media. Variance of $\delta^{34}\text{S}$ values was analyzed to determine source of sulfate minerals. Oxidation of minor amounts of pyritic coal enriched the $\delta^{34}\text{S}$ value in the brown plots. Overall, plots had acceptable water quality parameters, substantiating various types of mine spoils for the FRA, although brown proved best for tree establishment.

Methods were employed to determine whether *Phytophthora* were present in the surface of mine spoils and in infiltrated waters of 2005 plots as well as 2007 plots to determine if a chronosequential effect occurred. *P. cryptogea* was detected from surface spoil and from waters infiltrating brown sandstone plots. The brown spoil, relative to other spoil types, has greater soil moisture, greater nutrient availability, and lower pH, which may promote the occurrence and survival of the microorganism. The occurrence of the pathogen in the 2005 plots versus 2007 plots is notable; greater ground cover from colonizing species may be a precursor to *Phytophthora* detection on the plots. *P. cryptogea* is a possible threat to American chestnut, however, high infiltration rates in loose-dumped mine spoils should reduce damage by the pathogen.

Over time, the relation between water quality parameters (as influenced by spoil matrix composition), tree success, and presence of *Phytophthora*, is of interest as certain

hydrogeochemical parameters may cause stress on trees that may increase susceptibility of plants to disease caused by *Phytophthora*. Alternatively, certain water geochemical parameters may directly affect *Phytophthora* by promoting or inhibiting survival and transport of the pathogen in spoil and infiltrated spoil waters; this too has consequences for tree establishment on loose-dumped mine spoils.

KEYWORDS: American chestnut, isotope, reforestation, hydrology, reclamation.

Kathryn M. Ward
January 28, 2009

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REFORESTED MINE LANDS IN APPALACHIA

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THESIS

Kathryn M. Ward

The Graduate School
University of Kentucky
2009

MATRIX GEOCHEMISTRY AND *PHYTOPHTHORA* OCCURRENCE ON
REFORESTED MINE LANDS IN APPALACHIA

THESIS

A thesis submitted in partial fulfillment of the
Requirements for the degree of Master of Science in the
College of Arts and Sciences
at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2009

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To Biscuit and Dewey

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CHAPTER 1

SULFATE AND CARBONATE GEOCHEMISTRY OF WATERS INFILTRATING LOOSE-DUMPED MINE SPOILS

1.1 INTRODUCTION

Prior to the Surface Mining Control and Reclamation Act of 1977 (SMCRA) (Public Law 95-87), reforestation was the reclamation technique of choice in the eastern U.S.; however, since its passage a steady decline in the amount, diversity, and productivity of forestland in all coal-producing areas of the country has been observed. When attempts were made to reforest post-SMCRA sites, high seedling mortality, slow growth, and poor production typically occurred due to highly compacted soils with inappropriate chemical characteristics (Graves et al., 2000). After the passage of SMCRA, coal operators more often chose hayland or wildlife habitat instead of forestland for revegetation due to bond release expediency and satisfaction of regulatory requirements concerning sedimentation and erosion control (Burger and Torbert, 1997). Until recently, most landowners and mining companies viewed tree planting on post-SMCRA mined lands as a waste of time because of the poor results of early reforestation efforts (Ashby, 1998). Although the obstacles to reforesting reclaimed surface mined sites are formidable, enhanced research and extension efforts and the development of the Forestry Reclamation Approach (FRA) in recent years have resulted in a “reforestation renaissance” in Appalachia.

Based on ecological, economic and cultural incentives for reforestation of mined areas, and the existing data on studies of tree growth on end-dumped tailing media, the United States Department of Interior’s Office of Surface Mining Reclamation and Enforcement (OSMRE), in association with the seven state regulatory authorities in the Appalachian Region, advocates the following forestry reclamation techniques, or FRA: (1) create a suitable rooting medium for good tree growth that is no less than 1.2 meters deep and comprised of topsoil, weathered sandstone and/or the best available material; (2) loosely grade the topsoil or topsoil substitutes placed on the surface to create a non-

compacted growth medium; (3) use native and non-competitive ground covers that are compatible with growing trees; (4) plant two types of trees – early succession species for wildlife and soil stability, and commercially valuable crop trees; and (5) use proper tree planting techniques (OSMRE, 2006). Mine reclamation using FRA seeks to establish forests in order to provide erosion control, promote better water quality for watersheds, and to grow hardwood species as a valuable renewable resource. Watershed quality, including aquatic life, has been shown to be adversely affected by coal mining practices in Appalachia (Pond, 2004; Hartman, 2005). The most significant factor for disturbance to benthic macroinvertebrate communities is specific conductance levels above 500 $\mu\text{S}/\text{cm}$, which correlate well with dissolved sulfate levels (Pond et al., 2008).

Previous research on mined lands has shown that loosely graded topsoil, weathered sandstone and/or other non-toxic topsoil substitutes are suitable growing media for establishing native hardwood forests in Appalachia (Sweigard et al., 2007a and b). Reclamation practitioners, however, have expressed confusion as to what constitutes the best available material other than topsoil. Researchers have reported on the attributes of loose-graded brown weathered sandstone spoil compared to unweathered fine-textured rocks (Torbert et al., 1990). Preferably, the brown weathered sandstone should be derived from the top 3 meters of the soil profile but may be from below the top 3 meters if unavailable. Brown sandstone may be mixed with up to two-thirds of the “best available material” (Federal Register, Vol. 65, No. 161, 18 Aug 2000, 50409-50431). Other studies have identified certain types of loose-graded, mixed, unweathered sandstone and shale (mine run) spoil as being highly productive (Conrad et al., 2002; Angel et al., 2006). Recently, Angel (2008) concluded that the brown sandstone spoil had a higher productivity potential for trees and natural regeneration due to a finer texture and more suitable pH than gray sandstone or mixed sandstone and shale spoils. However, matrix geochemistry and inherent qualities of brown spoil material may also present challenges with regards to water quality and tree establishment. Although the mine spoil waters have exhibited water quality measurements that fall within EPA standards for treated water or secondary water regulations, Angel (2008) noted that infiltrated water from gray sandstone, mixed sandstone and shale and one brown sandstone plot exhibited a steady decline in dissolved ion concentration over time, while a second brown sandstone plot

exhibited an increase in dissolved ions over the same period. In particular, dissolved sulfate concentrations were increased in the second brown sandstone plot. The source and type of reaction driving up sulfate concentrations were unclear.

The selection of spoils and growth of trees and other vegetation in mine spoils reclaimed using FRA must be analyzed extensively over a period of time to determine how mine spoil choice and vegetation influence water quality. The setup of the Bent Mountain surface mine reforestation project in Pike County, Kentucky, provides a unique opportunity to quantify temporal variation of water-quality attributes of various tailing media with regards to reforestation efforts. Analysis of geochemistry and water-quality data of waters that had infiltrated through two replicates of brown weathered sandstone, gray unweathered sandstone, and mixed sandstone and shale spoils for the past three growing seasons (Angel, 2008) is necessary to determine factors influencing the suitability of spoil types for the reclamation process, and may lead to the omission, favor, or mixture of spoil types.

To gain a better understanding of what matrix minerals were most contributing to aqueous geochemistry, speciation and saturation indices were calculated for carbonate, sulfate, and other mineral phases using PHREEQC (Parkhurst and Appelo, 1999) from water samples collected at various sampling intervals. In addition, bivariate plots of various cations and anions were examined to better understand the dissolution of various minerals as controls on general water chemistry. To isolate the source of dissolved sulfate in spoil waters, sulfur isotopes were analyzed. Sulfur isotope values from dissolved sulfate in water samples, along with other water quality measurements, have been useful in past studies (for example, Mayo et al., 1992) for delineating source and nature of elevated sulfate levels and have led to a better understanding of the matrix weathering process. In particular, these values may help to delineate between dissolution of sulfates and oxidation of pyrite, each of which should have a unique $\delta^{34}\text{S}$ value. The values of $\delta^{34}\text{S}$ are expected to vary for the various rock types found in gray, brown, and mixed mine spoil, and therefore, dissolved sulfate from each respective spoil type should have a unique isotopic signature. Typical values of $\delta^{34}\text{S}$ derived from mineral constituents of various rock types of Paleozoic age, including formations mined for coal in Eastern Kentucky, are discussed. In addition to infiltrated mine spoil waters, $\delta^{34}\text{S}$ values were

determined for waters from alkaline and acid mine drainage sites within southeastern Kentucky.

1.2 STUDY AREA

The study site was located at the Bent Mountain strip mine in Pike County, Kentucky (latitude N 37° 35' 49", longitude W 82 ° 24' 19") operated by Appalachian Fuels (Fig. 1.1). Bent Mountain is in the Cumberland Plateau physiographic region and the Hazard Coal Reserve District as defined by the U.S. Geological Survey (Huddle et al., 1963). At the site, the Lower and Middle Pennsylvanian Breathitt Group, consisting of shale, siltstone, argillaceous and lithic sandstone, and some thin limestone, is mined for coal. The land is classified as mixed mesophytic forest and Appalachian oak forest. The principal soil order is Ultisols (USDA, 1998) and at the study site, the Dekalb soil series is present on upper side slopes and ridges (Hayes, 1982). Climate is humid and temperate, with temperatures reaching highs and lows of 32 and 18° C during the summer and 7 and -4° C during the winter. Average annual rainfall is 114 cm.

The loose-dumped mine spoils in this study were derived from the Lower and Middle Pennsylvanian Breathitt Group. Generally, the sandstone is light gray, massive, fine to medium grained, and weathers to a yellowish or reddish brown. The shale is dominantly medium gray, silty, and contains siderite nodules (Chesnut, 1992; Wolcott and Jenkins, 1966). The formation contains more than seven coal seams that have been mined (Permit 898-0056 Amendment No. 2, DSMRE ID 000339, 2001) at the site.

Six research plots to evaluate the performance of trees on three loose-graded spoil types were created in 2005 (Fig. 1.2), consisting of two replicates of 1) brown weathered sandstone, 2) predominantly gray, unweathered sandstone and 3) equally mixed brown weathered sandstone, gray unweathered sandstone, and shale (mine-run spoil). Plots are approximately 63 meters on each side and cover an approximate area of one acre or around 4,050 square meters. The spoil types were "end dumped" in large, parallel rows 2-3 meters deep. The parallel ridges were "struck-off" with one pass of a small bulldozer (Caterpillar® D-5) down the length of the ridge, creating a rough, non-uniform surface with parallel valleys as specified in Reclamation Advisory Memorandum Number 124 issued by the Kentucky Department of Natural Resources (KDSMRE, 1997).

Each plot is isolated from the others by a 2.5-meter buffer zone and drains into its own sample monitoring station by means of lysimeters and PVC pipes. There are three 4.6-meter-square lysimeters in each plot that are drained to the exit points by PVC pipes nominally 2.5 centimeters in diameter (Figure 1.3).

Four species of trees, white oak (*Quercus alba*), red oak (*Quercus rubra*), yellow poplar (*Liriodendron tulipifera*) and green ash (*Fraxinus pennsylvanica*), were planted on a 1.8 m x 2.4 m spacing onto the six plots in April 2005. In the spring of 2006, American chestnut (*Castanea dentata*) seedlings were planted on each of the six plots on one sub-plot measuring 7 m x 7 m. Each sub-plot consists of approximately 23 container-grown seedlings with 1.5 m x 1.5 m spacing.

1.3 METHODS

1.3.1 DATA COLLECTION

From May 2005 to February 2007, electrical conductivity, pH, Cl^- , SO_4^{2-} , Mg^{2+} , Ca^{2+} , K^+ , Na^+ , NO_3^- -N, NH_4^+ -N, total carbon (TC), total organic carbon (TOC) and dissolved organic carbon (DOC) were measured. Analysis of nitrate (NO_3^- -N), and ammonium (NH_4^+ -N) was performed by colorimetric analysis using a Bran+Luebbe Autoanalyzer (Bran+Luebbe, Analyser Division, Germany). Continuous-flow multi-test methods for NO_3^- -N and NH_4^+ -N (MT7/MT8 (EPA 353.2) and MT15/16 (EPA 350.1) respectively, were used. Sulfate (SO_4^{2-}) and chloride (Cl^-) concentrations were quantitated on a Dionex Ion Chromatograph (IC) 2000 (Dionex Corporation, California). Measurements of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) concentrations were made with a GBC SDS 270 Atomic Adsorption Spectrophotometer (AAS) (GBC Scientific Equipment, Hampshire, IL).

TOC and DOC were measured on samples of ≤ 2 mL with a Shimadzu TOC 5000A Analyzer (Shimadzu Corporation, Columbia, MD). Samples for the measurement of TOC were unfiltered, while DOC samples were filtered prior to analysis. TOC and DOC were calculated as the difference between total carbon (TC) and inorganic carbon (IC). Calibration curves were validated using a TC standard solution (reagent grade potassium hydrogen phthalate in “zero water” [carbon free water]) and an IC standard

solution (reagent-grade sodium hydrogen carbonate and sodium carbonate in “zero water”). For TC, samples were combusted at 680°C with high-purity air as the carrier gas to CO₂. A non-dispersive infrared gas analyzer (NDIR) is used to detect the CO₂. The NDIR generates a peak, whose area is proportional to the TC concentration of the sample. IC concentration is determined in the same procedure as the TC concentration, except the IC sample is injected into an IC reactor vessel where it is acidified to decompose the sample.

Additionally, waters collected in November 2006 and April-May 2008 were analyzed for aluminum (Al), barium (Ba), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), silicon (Si), strontium (Sr), and zinc (Zn) by inductively coupled plasmaoptical emission spectroscopy (ICP-OES) (Varian Vista-Pro CCD Simultaneous, Palo Alto, CA). Alkalinity and dissolved oxygen measurements were made for April-May 2008 waters using a Hach kit.

1.3.2 PHREEQC GEOCHEMICAL MODELING

PHREEQC geochemical modeling was used to model data collected the last three growing seasons, including biweekly data from analyses of major cations and anions, and ICP sampling data from November 2006 and April-May 2008. Speciation of sulfate minerals, carbonates, and other minerals of interest was analyzed (Parkhurst and Appelo, 1999). Saturation indices of minerals may be used to predict reactive minerals in solution. However, it must be kept in mind that due to short residence times in the loose-dumped mine spoils (on a scale of days), mineral-water equilibrium (on a scale of days to 10⁶ years) may not necessarily be achieved (Eby, 2004). Saturation indices above -10 were reported. A saturation index of 0 indicates equilibrium; for practical purposes, indices within the range of -0.5 to 0.5 are generally considered to be in equilibrium due to various sources of error (Deutsch, 1997). Saturation indices above this range, i.e. oversaturation, may indicate that the mineral is not reactive in the system. Percent error was also noted ($\text{percent error} = 100 * (\text{cations} - |\text{anions}|) / (\text{cations} + |\text{anions}|)$); in general, less than 5% error is considered acceptable. Larger percent errors are common nonetheless, especially when positive percent errors result from underestimate of anions. Alkalinity

measurements for data prior to 2008 were estimated to provide error under 1% as this parameter had not been measured.

1.3.3 SULFUR ISOTOPES

Sulfur was extracted by precipitation of BaSO₄. Sulfur isotope samples were collected in 2-L bottles and filtered through a 0.45-μm filter in the lab. To drop the pH below 5, 4 mL concentrated HNO₃ was added to each 1-L sample. In a fume hood, 0.4 M BaCl₂ (10%) solution was added by pipette until a white precipitate formed. The solution was allowed to precipitate and settle overnight. The precipitate was scraped off the bottom with a glass stir rod. The most precipitate was observed on the second brown sandstone plot, hereafter referred to as brown-2. The samples were filtered through a 0.45-μm filter. The filters were dried at 80°C for 1 hour. The sample was pulverized in a ceramic mortar dish and stored in a 2-mL centrifuge tube (Galvin, 2006).

Additionally, a grab sample of pyritic coal collected from the brown-2 plot was dissolved in deionized water. This coal sample was tested by X-ray diffraction and X-ray fluorescence for the presence of pyrite and other sulfide minerals and for bulk sulfur content. XRD analysis was carried out at the Kentucky Geological Survey Laboratory using a Bruker-AXS D8 DISCOVER Diffractometer (Karlsruhe, Germany). Reference samples included acid mine drainage sites in Pulaski and Laurel County as well as alkaline mine drainage sites from Robinson Forest in Perry County. These reference samples were tested in the field for pH, EC, and temperature and in the lab by ICP-OES as the other samples.

Analysis of ³⁴S took place at the University of Arizona's environmental isotope lab using a stable isotope mass spectrometer. Results are reported in the conventional δ notation,

$$\delta^{34}\text{S}, \text{‰} = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000,$$

where R is the ³⁴S/³²S isotopic ratio and the reference material is Vienna Cañon Diablo Troilite (VCDT).

1.4 RESULTS

1.4.1 GENERAL WATER QUALITY

In general, water quality parameters such as electrical conductivity have decreased with time, indicating that most plots are reaching equilibrium values after four years of emplacement and tree growth. The water chemistry of all plots is dominated by carbonate dissolution, with silicate weathering also playing a minor role. The brown plots, especially brown-2, exhibit EC and sulfate levels that have increased with time, suggesting that weathering is ongoing.

Table 1.1 demonstrates various physical and chemical characteristics of the Bent Mountain spoil waters as well as acid and alkaline mine drainage waters. Gray-2 did not yield water samples as no water was flowing during the sampling interval, indicating that a blockage in the pipes and/or leakage to another area had developed in the plot. Dissolved oxygen measurements (not shown) were comparable for the mine spoil plots and ranged between 7 and 8 mg/L, indicating highly oxidizing waters. Note the significant differences between the brown plots for EC, alkalinity, $\delta^{34}\text{S}$ value, and sulfate concentrations. pH values for waters infiltrating through the plots were in general lower for the brown-1 plot. Mixed plots were similar in the parameters measured, and the gray plots had previously been measured as good replicates in physical and chemical characteristics. Acid and alkaline mine drainage waters exhibited pH values between 2.92-3.43 and 6.70-7.22, respectively. Alkaline mine drainage had high sulfate values, around 1200 mg/L, whereas acid mine drainage waters ranged from 38 to 453 mg/L of dissolved sulfate.

A plot of dissolved calcium versus bicarbonate indicates a 1:1 correlation between dissolved calcite and bicarbonate for the brown spoils, but not for mixed and gray spoils during April-May 2008 (Figure 1.4). This may indicate that calcite is being dissolved at a greater rate in the brown spoils. Bicarbonate is released from silicate mineral weathering. When calcium plus magnesium versus bicarbonate are plotted (Figure 1.5), most plot along a 1:1 dissolution line, but not the brown-2 plot. The source of magnesium is likely chlorite, illite, or gibbsite as dolomite is rare in the Breathitt Formation (Papp, 1976).

Plots of sodium versus chloride concentrations are useful in examining the effects of Cl^- from rainwater and Na^+ from silicates. A 1:1 equimolar line is plotted where dissolution of halite, NaCl , would occur (Figures 1.6 and 1.7). All mine waters are undersaturated in halite; saturation indices for halite ranged from -8 to -9 for all plot waters. The samples were diluted in Cl^- with respect to Na^+ . The weathered, brown spoils have the greatest amounts of both Na^+ and Cl^- , although Cl^- is typically flushed from weathered rocks. Cation exchange processes may have acted to adsorb Na^+ to clay minerals in the brown sandstone after being exchanged with C.

Graphs to evaluate stability of Ca-Al and Na-Al silicate phases were constructed to determine roles of various clay minerals in influencing water chemistry (Figures 1.8 and 1.9). All samples plot in the kaolinite field, except gray-1 which is in the gibbsite stability field. These clay minerals had been identified by whole-rock XRD analysis (Angel, 2008) for all three spoil types.

Na^+ -normalized molar diagrams (Figures 1.10 and 1.11) show that carbonate weathering and a minor amount of silicate dissolution are general controls on water chemistry. Brown-2 has slightly more influence from silicate weathering versus the other samples for both Mg^{2+} and HCO_3^- normalized plots.

1.4.2 SULFATE

Sulfate levels were measured from July 2005 to May 2008. The levels oscillated distinctly at the onset of the study and have become more stable since the first year of emplacement. In particular, the sulfate levels of the mixed and gray plots have decreased and stabilized, but the sulfate levels of the brown plots have increased (Figure 1.12). The noticeable decline in elevated sulfate concentrations after June 2006 for the gray and mixed spoil plots may be associated with the removal of smaller particles of pyrite by weathering processes. The brown plots demonstrated overall higher total dissolved solids (TDS) contents that increased with time, indicating greater weatherability for this spoil type. Electrical conductivity measurements also displayed marked fluctuations at the onset of the study and decreased with time with the exception of the brown spoils (Angel, 2008).

The two brown plots exhibit an overall increase in sulfate levels that began around July 2006 (Figure 1.13), indicating they have not yet reached equilibrium. There is a general difference of approximately 400 mg/L between the plots, with brown-2 having higher amounts of sulfate as well as more pronounced fluctuations.

Graphs of calcium versus sulfate were plotted for water samples collected during May 2006 and April-May 2008 (Figures 1.14 and 1.15). Note that most of the plots fall above the gypsum dissolution line, which is marked by equimolar proportions of Ca^{2+} and SO_4^{2-} , indicating that some reaction is adding excess SO_4^{2-} , consuming Ca^{2+} , or both. After most of the plots decreased in sulfate levels from May 2006 to April-May 2008, they plot near the calcium to sulfate dissolution line. However, brown-1 and brown-2 plots are higher in sulfate levels. Brown-2 plots well above the 1:1 line; therefore, a non-gypsum source, likely oxidation of pyrite, must be present to raise sulfate levels and drive calcium levels up (buffer effect). This may be happening to a much lesser extent in the brown-1 plot and gray-1 plot as these waters plot slightly above the 1:1 line.

Dissolved calcium levels (Figure 1.16) have decreased with time for mixed and gray plots, from approximately 150 mg/L to below 50 mg/L, remained stable for the brown 1 plot at approximately 75 mg/L, and have been on the rise for the brown-2 plot, which measured at 204 mg/L in May 2008. Ca^{2+} has decreased with time for mixed and gray plots and been stable for brown plots (Angel, 2008), indicating it is likely being driven up by this process.

1.4.3 PHREEQC MODELING

Speciation of mineral phases and degree of saturation were evaluated for the mine spoil waters (Tables 1.4 and 1.5). The water chemistry of infiltrated waters of the Bent Mountain spoils is dominated by dissolution of carbonates, resulting in alkaline waters. Calcium and magnesium carbonates are the primary carbonates which likely occur as intergranular cements in the spoil. Calcite is the most neutralizing carbonate, followed by dolomite (Table 1.2). Carbonate saturations differed mostly between the brown-1 plots and the rest of the plots in 2006 and April 2008. The brown-1 plot is near equilibrium for calcite and dolomite, whereas the others are oversaturated. Saturation for carbonates was reached in May 2008 for the brown-1 plot. This observation corresponds with lower

alkalinity and pH values for the brown-1 plot. The lower pH values are the likely cause for equilibrium levels in the brown-1 plots as at higher pH values, carbonates are less reactive (Plummer et al., 1978; Stumm and Morgan, 1996).

Sulfate species evaluated in PHREEQC include anhydrite, gypsum, celestite, and barite, which are all secondary minerals that dissolve without producing acidity. Table 1.3 lists secondary sulfate minerals common in mine spoils in order of acid-producing potential. Sulfates containing aluminum, magnesium, and iron generally contribute to acidity through production of H^+ by hydrolysis, whereas the sulfates epsomite, gypsum, and barite do not. Sulfate minerals such as K-jarosite ($KFe_3(SO_4)_2(OH)_6$) and melanterite ($FeSO_4 \cdot 7H_2O$) that contribute to acidity were speciated for brown-2 and mixed-1 during April 2008. The mine spoil plots are undersaturated with respect to these sulfate species, indicating that these minerals are slow to dissolve under the given conditions, and therefore not highly significant in influencing water chemistry. Notable differences for saturation indices of anhydrite and gypsum are noted between brown-2 and the other plots. Brown-2 is nearest to equilibrium for these sulfate minerals, indicating that they are the most reactive in this plot. However, brown-2 had plotted well above the gypsum dissolution line on the bivariate plot of Ca^{2+} to SO_4^{2-} , so another source of sulfur is highly likely. Celestite ($SrSO_4$) was undersaturated in all spoil waters. Barite was near equilibrium in November 2006 waters and not identified in April-May 2008 except for brown-1 water samples, where it also was near equilibrium. Epsomite, $MgSO_4$, was not modeled, but was expected to have higher saturation index values than gypsum and anhydrite given the greater amount of dissolved magnesium.

Clay minerals can buffer acidity and drive up pH, as well as indicate weathering processes. The clay minerals chlorite and illite are common in Breathitt Formation rocks and are primary contributors to Mg^{2+} in solution (Papp, 1976). Difficulty in measuring Al concentrations may be affected by the occurrence of Al as colloidal particles ($<0.45 \mu m$) rather than in solution. For these minerals, activity-activity diagrams were also plotted (section 1.4.1).

Saturation indices of quartz relative to amorphous silica are consistent with amorphous silica being the phase controlling SiO_2 precipitation (Hem, 1992).

The presence of dissolved iron in brown-2 and mixed-1 plots on April 1, 2008 is unusual given the high pH of these samples, 8.13 and 8.27, respectively. Although the samples were filtered, colloidal particles rather than dissolved iron may be the source. Nodules of siderite are present in mine spoils, but this iron carbonate is very insoluble at high pHs. Saturation indices for goethite, FeOOH , are above 7 for these two samples.

1.4.4 SULFUR ISOTOPES

$\delta^{34}\text{S}$ values were obtained for the majority of tested waters; however, in some cases, not enough barium sulfate was successfully precipitated from the sample. Analytical precision of $\delta^{34}\text{S}$ values was $\pm 0.15\%$. The value for a grab sample of pyritic coal from the brown-2 plot was 39.5‰. Phases of sulfur in coal include sulfide minerals, organic compounds, and minimal amounts of sulfate minerals. Pyrite was identified in a grab sample of pyritic coal (Figure 1.18). According to XRF analysis, the coal sample contained sulfur as approximately 50% pyrite and 50% sulfate.

Figure 1.17 depicts the sulfur isotopic signature for each water sample versus its amount of dissolved sulfate. Acid mine drainage waters, Wildcat Branch and Bear Creek, as well as alkaline mine drainage waters, Guy Cove and Bee Branch, are plotted for reference. Each mine spoil plot had stable $\delta^{34}\text{S}$ values during the sampling interval, despite fluctuations in sulfate measurements. Within the spoil plots, a positive correlation is evident between $\delta^{34}\text{S}$ value and dissolved sulfate. The mixed sandstone and shale plots have $\delta^{34}\text{S}$ values below -2.0‰; shale is the main source of dissolved sulfate in these plots and likely was depleted in $\delta^{34}\text{S}$ due to bacterial reduction of sulfate during deposition (Kehew, 2001). The rate of bacterial SO_4^{2-} reduction and amount of seawater intrusion are factors controlling the $\delta^{34}\text{S}$ value (Elswick et al., 2007, and references therein).

Gray-1 and brown-1 are similar in sulfate and $\delta^{34}\text{S}$ values. Brown-2 has higher levels of sulfate and a higher $\delta^{34}\text{S}$ value, indicating a different source of sulfate than the other plots. Pyritic coal in this plot, with a much higher $\delta^{34}\text{S}$ value given the grab sample from this plot, has likely oxidized to increase sulfate levels and the $\delta^{34}\text{S}$ value. Brown-2 has high alkalinity values, up to three times that of the brown-1 plot. This is likely due to an acid-base accounting issue: the factor driving carbonate dissolution is the oxidation of a greater amount of pyrite in brown-2 than brown-1.

The acid mine waters with sulfate levels attributed to pyrite oxidation plot closest to the infiltrated mine spoil waters. The acid mine drainage site Wildcat Branch plots fairly close to brown-1 and gray-1 data. Bear Creek, however, had a much higher $\delta^{34}\text{S}$ value. Alkaline mine waters, Guy Cove and Bee Branch, were similar in isotopic signature but much greater in sulfate concentrations than the mine spoil waters. Although dissolved sulfate levels and other hydrochemical characteristics were similar, there were discrepancies between $\delta^{34}\text{S}$ values of alkaline drainage waters (0.1 and -4.6). These values may indicate the source of dissolved sulfate was a mineral with a relatively broad range of $\delta^{34}\text{S}$ values. Acid mine drainage sites varied in $\delta^{34}\text{S}$, which was seemingly indirectly correlated with dissolved sulfate.

1.5 DISCUSSION AND CONCLUSIONS

Loose-dumped mine spoils of various types, emplaced using the forestry reclamation approach, demonstrated overall good infiltrated water quality from 2005 to 2008. Water chemistry is dominated by the dissolution of carbonate minerals, resulting in a high buffering capacity of the system. Among spoil types, variances in dissolved solutes were influenced by matrix composition and weathering capacity. The factor controlling alkalinity production and sulfate dissolution in the spoils is spoil matrix composition, especially amounts of sulfate and pyrite in coal incorporated into the spoils; spoil matrix composition in turn influences weatherability. Elevated sulfate levels in the brown plots were confirmed by isotopic data to be derived from pyritic coal seams. However, alkalinity of the spoils is sufficient to prevent acid rock drainage.

Certain factors that influence reaction rates within loose-dumped mine spoils, including oxygen diffusion, infiltration rate, and compaction, are all comparable between plots (Taylor et al., 2008; Angel, 2008). However, reaction rates differ between the brown sandstone spoils and other spoil types due to pyrite and carbonate mineralogy of the spoils, as well as amounts of these minerals. Additionally, weatherability of the brown plots increases reaction rates; weatherability is significantly higher as evident by rock durability analyses (Angel, 2008). Analysis by X-ray diffraction on all mine spoils failed to detect pyrite, which is not surprising as sampling error is high given the amount

of mine spoil needed for a representative sample. Non-random sampling was needed to confirm pyritic coal from the brown-2 plot (Fig. 1.14).

Sulfur isotopes from dissolved sulfate in mine spoil varied with spoil type. Sources of sulfur in the types of mine spoil may include pyrite and organic sulfur found in shale and coal as well as secondary sulfate minerals that may precipitate near sulfides. An elevated $\delta^{34}\text{S}$ value, in conjunction with higher dissolved sulfate and alkalinity values, is good evidence for the oxidation of pyrite in coal and a buffering reaction occurring in the brown-2 plot, as well as dissolution of sulfate in coal. In brown-1, this reaction may also be occurring on a smaller scale, given that sulfate levels rose during the 2005-2008 sampling period. Bivariate plots of calcium versus sulfate have shown that the mixed plot and gray spoils plot near a 1:1 line, whereas the brown plots are elevated with respect to sulfate.

Average rock elemental concentrations for sulfur in mg/kg by rock type were measured in 2007 (Table 1.6). Brown sandstone, gray sandstone, and shale had sulfur levels of 0.23%, 0.25%, and 0.30%, respectively. The range of percent sulfur in seams mined at Bent Mountain is 0.58-3.31% (KDSMRE, 2001). Coal from one of the seams incorporated into the mine soils had 1.19% sulfur. Another sample from the Broas coal seam, which was only incorporated into the brown-2 plot, had a high percent sulfur (3.3%) and a high percent pyritic sulfur (0.9%) (Angel, 2008). Similarly, in this study, a grab sample of coal from brown-2 had approximately 3% sulfur, approximately half of which was pyritic sulfur. Brown-1 was derived from spoils approximately 75 m lower stratigraphically, near the Winfred coal seam, with a 1.01% sulfur content, which is known to contain lesser amounts of pyritic coal.

Sulfur isotope values vary between late Paleozoic minerals present in the mine spoil matrix at the Bent Mountain research area. Sulfur isotope values for Paleozoic marine evaporites range from +10 to +22‰ (Clark and Fritz, 1997). However, these evaporites are unlikely to remain in the strata. Sulfur derived from pyrite in coal may have a broad range of $\delta^{34}\text{S}$ values, from -20 to +50‰, depending on the amount of fractionation and mineralization of “heavy” sulfur to pyrite by bacterial SO_4^{2-} reduction, and interaction of the coal with seawater (Smith and Batts, 1974; Ryan and Ledda, 1997). Elswick et al. (2007) found that $\delta^{34}\text{S}$ values ranged from -1.09 to +31.66‰, with an

average value of +20.76‰, for the Dean coal bed of the Middle Pennsylvanian Hyden Formation, which is stratigraphically just above the Pikeville Formation. The mixed sandstone and shale spoils were slightly negative, whereas gray and brown sandstone spoils were slightly positive. The shales have a lower $\delta^{34}\text{S}$ value due to anoxic, marine influences (Clark and Fritz, 1997), which contributed to lower isotopic values for sulfates derived from the mixed and gray spoil waters. Pennsylvanian shales are variable in $\delta^{34}\text{S}$ value and may contain sulfides that are slightly to highly depleted (Coveney and Shaffer, 1988). The plot of $\delta^{34}\text{S}$ and dissolved sulfate likely represents an influence from more enriched, pyritic coal and more depleted shales. Sulfate levels in atmospheric precipitation are in the range of 1.5 mg/L (1995 est., National Atmospheric Deposition Program/National Trends Network, 1996). However, the input of sulfate from precipitation to mine water sulfate content is minimal and consistent between plots and therefore should not affect comparisons of the $\delta^{34}\text{S}$ values. Fractionation does not play a major role in influencing $\delta^{34}\text{S}$ value when sulfate is derived from sulfide oxidation (Taylor et al., 1984; Seal and Wandless, 1997; Balci, N. et al., 2007). The $\delta^{34}\text{S}$ value of sulfate derived from pyritic coal in this study was highly enriched, with a value of +39.5‰, which is likely from isotopic fractionation from sulfide minerals. Wunsch (1988) reported that the isotopic composition of surface waters with sulfates derived from sulfide mineral oxidation was well represented by a stream with value of +3.4‰.

The brown-2 plot differed from the brown-1 plot and from the other spoil types by its elevated alkalinity, elevated sulfate, greater $\delta^{34}\text{S}$ value, and higher electrical conductivity. The capability of this replicate to weather more, as evident by greater EC, may result in greater particle surface area and promote dissolution of the calcite matrix; in turn, reactivity with sulfate and sulfide minerals in coal increases. Elevated dissolved sulfate levels result, and the sulfur signature is enriched from sulfide or sulfates in coal. In addition, secondary sulfate minerals, including gypsum, are near equilibrium with the system, suggesting they are reactive and contributing to alkalinity. Brown-2 also contained greater percent pyritic coal than brown-1. The positive feedback generated by the oxidation of pyritic coal is the likely driving force behind the increasing alkalinity and sulfate levels in this plot. In comparison, brown-1 exhibited a slight increase in sulfate levels since 2005, but not calcium; it has lower pH, alkalinity, $\delta^{34}\text{S}$, and EC than brown-

2. However, rate of weathering was higher on both brown plots after three years versus the gray and mixed plots based on observations of settleable solids (Taylor et al., 2008).

The main control on alkalinity in the system was the dissolution of calcite, with minor dissolution of dolomite. As calcite is the most soluble of the carbonates, this is not unlikely. Calcite was previously reported as the main cementing agent for sandstone and shale mine spoils (Wunsch, 1996). Calcite dissolution contributes to alkalinity by producing HCO_3^- anions in solution. The rate of dissolution may be increased by greater weathering of spoil matrix which results in smaller particle size and hence larger surface area for reactions to occur. Infiltrated water pH, which may be driven down by oxidation of pyrite, also increases reaction rate as pH decreases. Oxidation of pyrite in the brown plots, especially brown-2, generates H_2SO_4 that could further dissolve calcite.

The main controls on dissolved sulfate are dissolution of pyrite and sulfate in coal. The sulfur isotopic signatures of the plots and their sulfate concentrations are in direct correlation. Brown-2 received sulfate contributions from pyrite and sulfate in coal evident by a sulfur isotopic signature that was slightly higher than the other plots. Continued monitoring of sulfate concentrations and isotopic variation of sulfur will shed more light on the reaction rates and dynamics of carbonate dissolution and pyrite oxidation in the infiltrated waters over time.

TABLE 1.1. Physical and chemical characteristics of Bent Mountain mine spoil waters, April-May 2008, and other waters in this study.

Sample	pH	EC	Alk.	$\delta^{34}\text{S}$ ‰	F ⁻	Cl ⁻	SO ₄ ²⁻	NO ₃ ⁻	Fe	Mg	Si	Sr	Al	B	Ca	K	Na	Charge balance
4-1-2008																		
BROWN 1	7.35	193	103.1	1.1	0.18	3.27	262.96	0.23	0.00	43.31	3.32	0.19	0.03	0.14	75.37	4.83	21.17	7.40e-004
BROWN 2	8.13	410	248.7	4.1	0.35	3.89	676.60	5.75	0.07	141.75	1.99	0.40	0.02	0.08	177.28	9.99	21.98	2.55e-003
MIXED 1	8.27	216	232.5	-3.0	0.18	1.38	182.25	7.22	0.11	72.56	1.15	0.27	0.04	0.07	60.43	15.39	4.70	1.10e-003
MIXED 2	8.36	178	228.5	-2.3	0.22	1.11	134.97	5.95	0.00	59.07	1.04	0.26	0.02	0.06	52.68	7.90	17.83	5.75e-004
GRAY 1	8.38	234	345.7	0.8	0.13	1.18	146.48	2.55	0.00	102.32	0.86	0.23	0.02	0.11	39.90	10.22	5.41	1.06e-003
4-14-08																		
BROWN 1	8.07	158	82.9	1.2	0.17	2.80	191.31	0.27	0.00	31.87	3.27	0.15	0.02	0.05	55.26	4.31	17.64	5.35e-004
BROWN 2	8.41	348	208.2	4.3	0.37	3.15	464.90	4.39	0.00	95.06	1.90	0.28	0.01	0.04	132.72	15.39	4.70	1.48e-003
MIXED 1	8.56	195	236.5	-2.9	0.12	1.03	121.12	2.32	0.00	90.17	0.82	0.20	0.01	0.04	36.09	7.90	17.83	5.42e-004
MIXED 2	8.50	180	192.1	-2.4	0.17	1.01	129.78	6.52	0.00	61.63	1.08	0.23	0.02	0.03	49.27	9.86	5.03	8.70e-004
GRAY 1	8.57	236	337.6		0.22	0.94	119.88	5.29	0.00	53.37	1.06	0.24	0.01	0.03	47.81	10.09	4.61	5.20e-004
5-7-2008																		
BROWN 1	8.01	*	119.3	1.3	0.18	2.93	262.45	0.19	0.00	40.53	3.41	0.19	0.01	0.02	69.00	4.82	19.08	-2.03e-004
BROWN 2	8.04	*	307.3	4.2	0.28	4.12	797.11	4.77	0.00	147.89	2.05	0.42	0.04	0.02	204.36	17.58	5.72	8.86e-004
MIXED 1	8.17	*	268.9	-2.9	0.23	1.58	210.36	5.16	0.00	79.50	1.30	0.29	0.02	0.02	68.69	13.48	23.66	8.39e-004
MIXED 2	8.16	*	252.7		0.31	0.98	118.69	3.53	0.00	62.49	1.06	0.29	0.02	0.06	58.89	12.16	8.31	1.09e-003
GRAY 1	8.31	*	345.7		0.20	1.12	155.23	1.88	0.00	103.32	0.97	0.24	0.03	0.03	42.40	12.24	5.76	1.14e-003

TABLE 1.1. (continued)

	pH	EC	Alk.	$\delta^{34}\text{S}$ ‰	F ⁻	Cl ⁻	SO_4^{2-}	NO_3^-	Fe	Mg	Si	Sr	Al	B	Ca	K	Na	Charge balance
Acid mine waters (6-2-08)																		
WILDCAT BRANCH	2.92	240	0	4.3	0.37	0.52	453.73	0.03	13.5 5	31.02	9.58	0.23	12.8 1		55.91	2.57	1.20	-1.88e- 003
BEAR CREEK	3.43	46	0	7.9	0.00	0.74	38.68	0.03	1.53	1.18	2.98	0.10	1.47		4.30	1.45	0.00	-2.71e- 004
Alkaline mine waters (5-28-08)																		
GUY COVE	6.74	423	22.2	-4.6	0.19	1.55	1155.6 7	0.00	0.00	212.71	5.03	0.54	0.07	156. 50	8.19	13.25	0.05	1.72e- 003
BEE BRANCH	7.22	475	26.3	0.1	0.17	2.39	1200.4 0	0.22	0.00	222.58	4.87	1.07	0.07	222. 77	16.31	25.68	0.01	5.66e- 003

*indicates parameter not measured

TABLE 1.2. Common carbonate minerals in mine overburden, listed in descending order of their capability to neutralize acid. Reproduced from Cravotta and Hilgar (2000).

MINERAL	CHEMISTRY
CALCITE	CaCO_3
DOLOMITE	$\text{CaMg}(\text{CO}_3)_2$
ANKERITE	$\text{Ca}(\text{Fe}, \text{Mg})(\text{CO}_3)_2$
MN-SIDERITE	$(\text{Fe}, \text{Mn})\text{CO}_3$
SIDERITE	FeCO_3

TABLE 1.3. Secondary sulfate minerals identified in western Pennsylvania mine spoil and overburden. Compiled by Brady et al. (2000) and references therein.

ACID-PRODUCING	
PICKERINGITE	$\text{MgAl}_2(\text{SO}_4)_4 \cdot 22 \text{H}_2\text{O}$
HALOTRICHITE	$\text{Fe}^{2+}\text{Al}_2(\text{SO}_4)_4 \cdot 22 \text{H}_2\text{O}$
ALUNOGEN	$\text{Al}_2(\text{SO}_4)_3 \cdot 17 \text{H}_2\text{O}$
COPIAPITE	$\text{Fe}^{2+}\text{Fe}^{3+}(\text{SO}_4)_6(\text{OH})_2 \cdot 20 \text{H}_2\text{O}$
COPIAPITE GROUP	$\text{Al}_{2/3}\text{Fe}^{3+}_4(\text{SO}_4)_6(\text{OH})_2 \cdot 20\text{H}_2\text{O}$ with Mg
COQUIMBITE	$\text{Fe}_2(\text{SO}_4)_3 \cdot 9 \text{H}_2\text{O}$
ROEMERITE	$\text{Fe}_2+\text{Fe}_{23+}(\text{SO}_4)_4 \cdot 14 \text{H}_2\text{O}$
*JAROSITE	$\text{KFe}_3+3(\text{SO}_4)_2(\text{OH})_6$
NON-ACID-PRODUCING	
GYPSUM	$\text{Ca}(\text{SO}_4) \cdot 2 \text{H}_2\text{O}$
EPSOMITE	$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$
BARITE	BaSO_4

* Jarosite is less soluble than the other acid-producing sulfate minerals.

TABLE 1.4. PHREEQC modeling, November 2006 data. Regular font signifies saturation index (SI) < -0.50; **pink** font signifies $0.50 \geq \text{SI} \geq -0.50$; **bold** signifies SI > 0.50.

11-17-06								11-17-06 WITH ALKALINITY EST.					
MINERAL		BROWN 1	BROWN 2	MIXED 1	MIXED 2	GRAY 1	GRAY 2	BROWN 1	BROWN 2	MIXED 1	MIXED 2	GRAY 1	GRAY 2
SULFATES	ANHYDRITE	-2.12	-1.42	-1.95	-1.95	-2.05	-1.90	-2.16	-1.45	-2.00	-1.99	-2.10	-1.95
	BARITE	-0.62	-0.53	-0.94	-0.77	-0.64	-0.78	-0.46	-0.36	-0.79	-0.60	-0.52	-0.65
	CELESTITE	-2.60	-1.82	-2.21	-2.19	-2.19	-2.02	-2.61	-1.84	-2.23	-2.21	-2.21	-2.03
	GYPSUM	-1.90	-1.20	-1.73	-1.73	-1.83	-1.68	-1.91	-1.20	-1.75	-1.74	-1.85	-1.70
CARBONATES	CALCITE							-0.33	1.02	0.76	0.83	0.81	0.98
	DOLOMITE							-0.57	2.41	1.91	2.03	2.16	2.39
	SMITHSONITE								-1.12	-1.74		-1.35	-1.17
	STRONTIANITE							-2.22	-0.81	-0.91	-0.83	-0.75	-0.55
	WITHERITE			-1.10		1.72		-4.27	-3.53	-3.67	-3.42	-3.26	-3.36
	AMORPHOUS SILICA	-1.52	-1.52	-1.93	-1.98	-2.02	-2.07	-1.43	-1.92	-2.42	-1.84	-1.89	-1.98
	QUARTZ	-0.25	-0.25	-0.66	-0.72	-0.75	-0.80	-0.10	-1.08	-0.50	-0.56	-0.59	-0.64
WILLEMITE			0.83			2.62		-1.47	-1.71		-1.13	-0.21	
PERCENT ERROR†		36.61	39.43	39.43	48.76	58.88	49.7	0.37	0.25	0.56	-0.70	0.03	0.82

†percent error=100*(cations-|anions|)/(cations+|anions|)

TABLE 1.5. PHREEQ-C modeling for April and May 2008 data. Regular font signifies saturation index (SI) <-0.50; **pink** font signifies $0.50 \geq \text{SI} \geq -0.50$; **bold** signifies $\text{SI} > 0.50$.

		4-1-4-01					5-07-2008				
SULFATES	MINERAL	BROWN 1	BROWN 2	MIXED 1	MIXED 2	GRAY 1	BROWN 1	BROWN 2	MIXED 1	MIXED 2	GRAY 1
	ANHYDRITE	-1.49	-0.96	-1.76	-1.92	-2.07	-1.53	-0.87	-1.69	-1.94	-2.04
	BARITE	-0.52					-0.91				
	CELESTITE	-2.12	-1.65	-2.16	-2.27	-2.34	-2.15	-1.62	-2.12	-2.31	-2.33
	GYPSUM	-1.23	-0.71	-1.51	-1.66	-1.81	-1.29	-0.65	-1.47	-1.73	-1.81
	K-JAROSITE		-2.91	-3.42							
	MELANTERITE		-7.89	-8.45							
CARBONATES	CALCITE	-0.43	0.95	0.73	0.92	0.76	0.36	1.17	0.92	0.86	0.90
	DOLOMITE	-0.95	1.98	1.69	2.24	1.76	0.75	2.53	2.25	2.09	2.49
	SIDERITE		-1.62	-1.62							
	AMORPHOUS SILICA	-1.43	-2.02	-1.66	-1.90	-1.96	-1.49	-2.06	-1.71	-1.90	-2.00
	QUARTZ	-0.07	-0.66	-0.31	-0.54	-0.61	-0.18	-0.77	-0.41	-0.59	-0.69
	GOETHITE		7.89	8.14							
	PERCENT ERROR	5.32	7.95	6.86	4.16	5.65	-1.51	2.56	4.81	7.55	6.08

TABLE 1.6. Average rock elemental concentrations in mg/kg by rock type in 2007. From Angel (2008).

ELEMENT	BROWN	GRAY	SHALE
Al	5928	5055	25030
Ba	26.1	23.5	93
Ca	797	7163	2851
Cr	57.1	70.7	41.9
Cu	2.8	4.8	24.1
Fe	13947	20961	42467
Mg	1000	3424	5827
Mn	112	377	720
Ni	7.6	6.8	30.2
K	1542	1677	5503
Na	91	88	258
Sr	4.8	16.3	54.7
Zn	21.6	25.3	114
S [†]	0.23	0.25	0.30

[†]Reported in percent sulfur. Range of sulfur in seams mined at Bent Mtn. is 0.58-3.31 (KDSMRE, 2001).

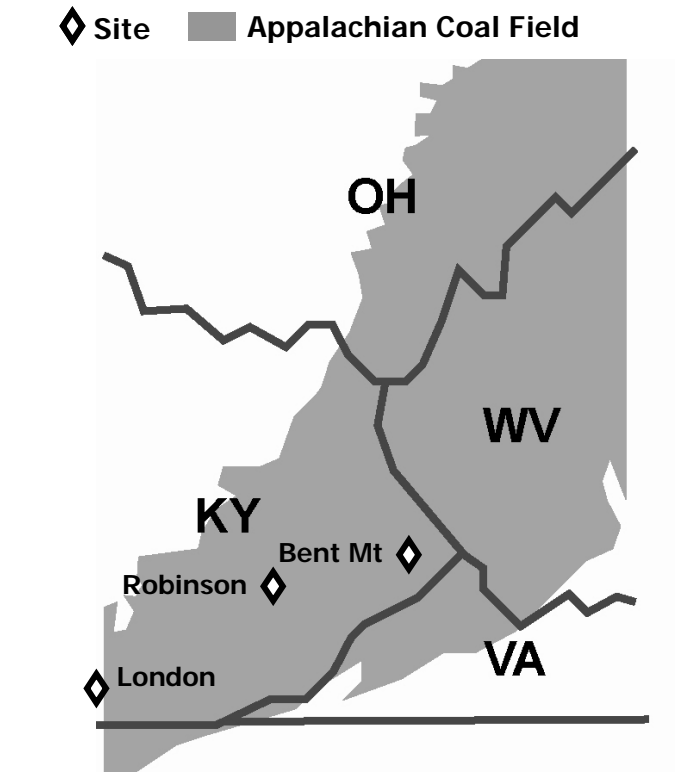


FIGURE 1.1. Location of study sites.



FIGURE 1.2. Aerial view of the 2005 loose-dumped plots at the Bent Mountain surface mine in Pike County, Kentucky. The size of each plot is approximately 0.4 hectare.

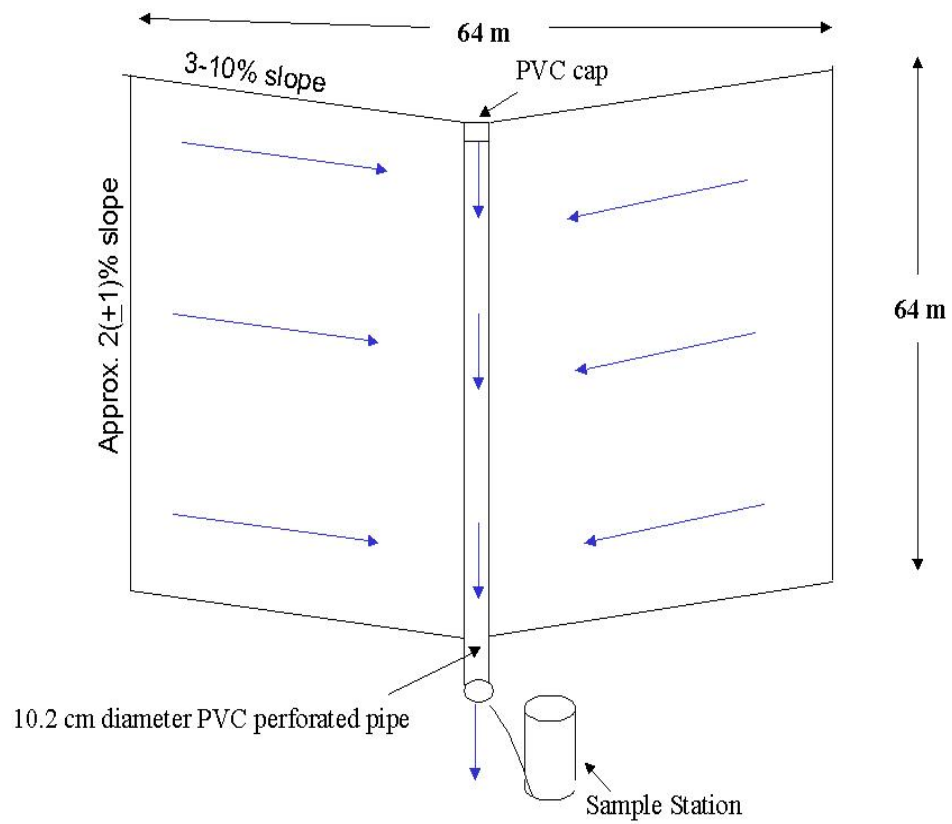


FIGURE 1.3. Schematic cross-section of loose-dumped mine spoil and infiltrated water collection system. Arrows indicate direction of flow.

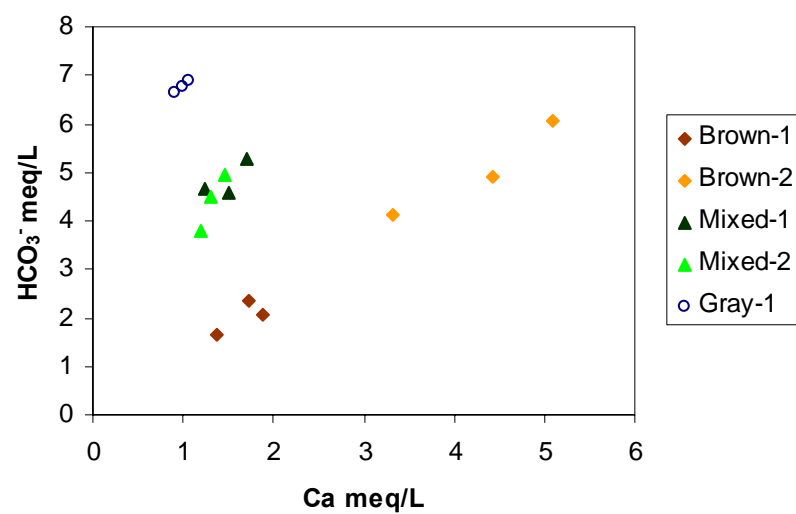


FIGURE 1.4. Plot of dissolved calcium to bicarbonate anions, April-May 2008 data.

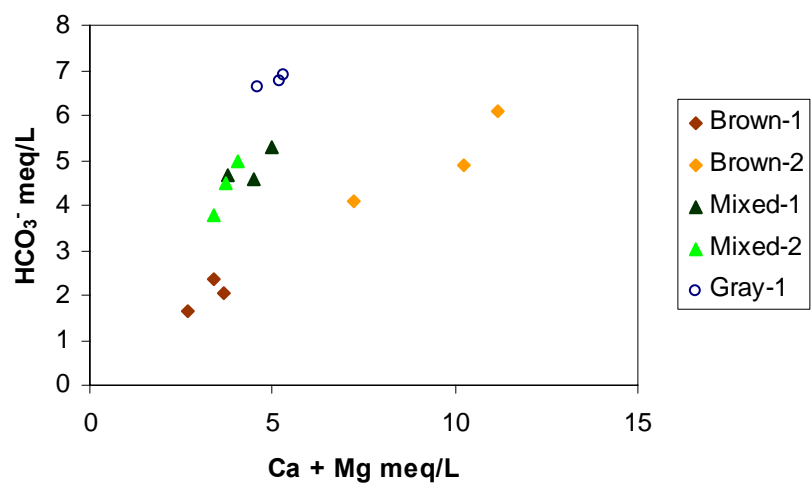


FIGURE 1.5. Plot of calcium and magnesium to bicarbonate anions, April-May 2008 data.

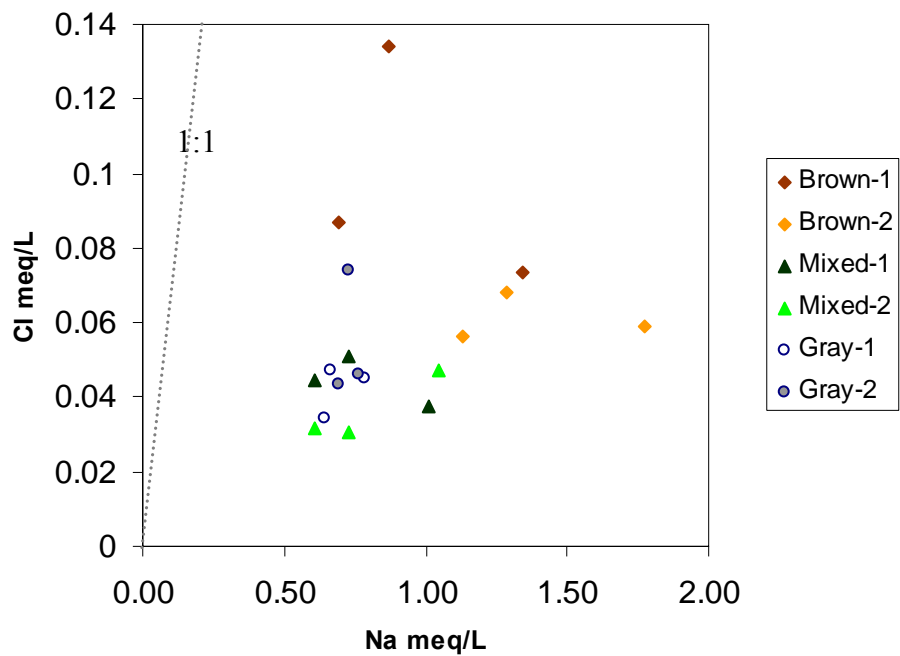


FIGURE 1.6. Plot of Na⁺ vs. Cl⁻ for Oct-Dec 2006 data.

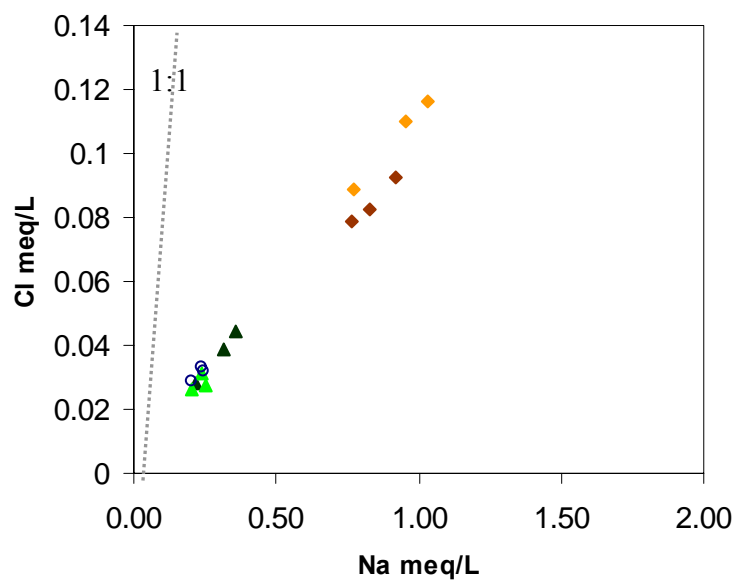


FIGURE 1.7. Plot of Na⁺ vs. Cl⁻ for April-May 2008 data.

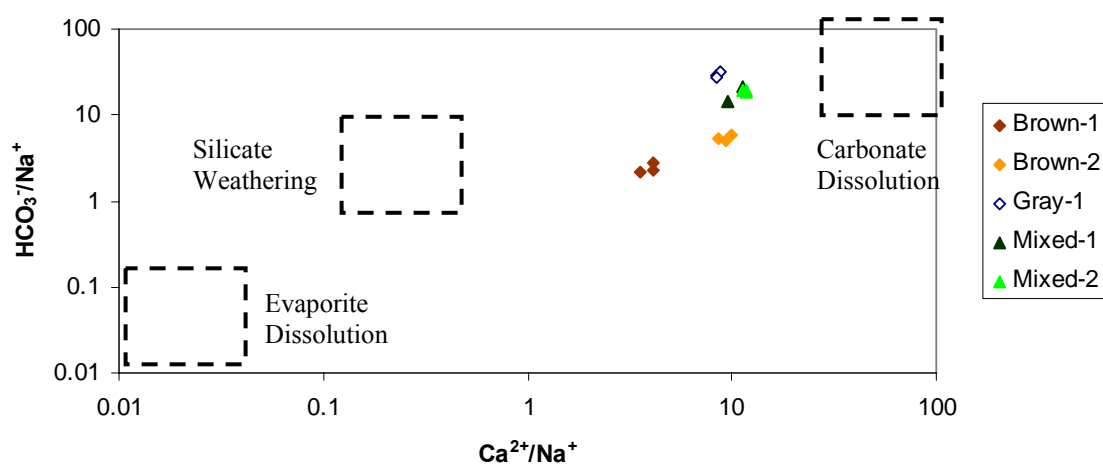


FIGURE 1.8. Molar ratio bivariate plot of Na^+ -normalized Ca^{2+} and HCO_3^- .

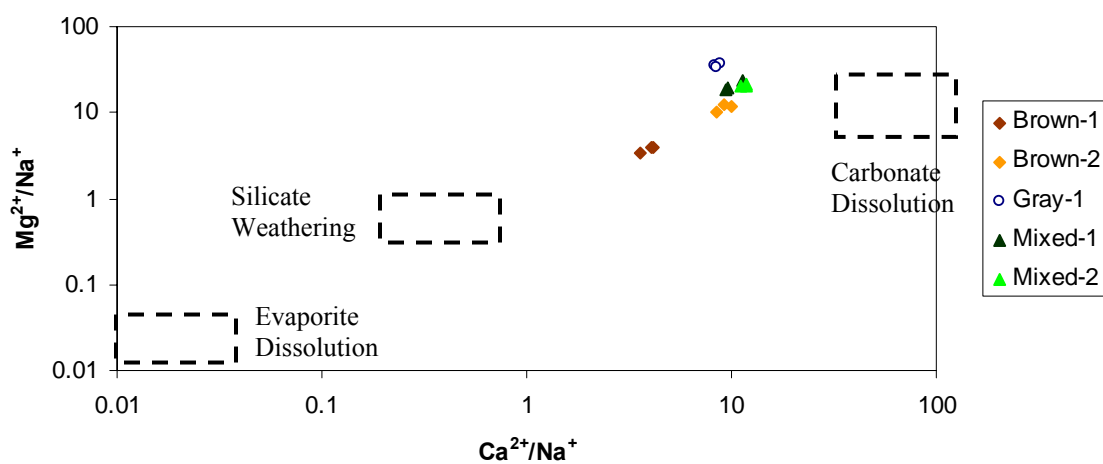


Figure 1.9. Molar ratio bivariate plot of Na^+ -normalized Ca^{2+} and Mg^{2+} .

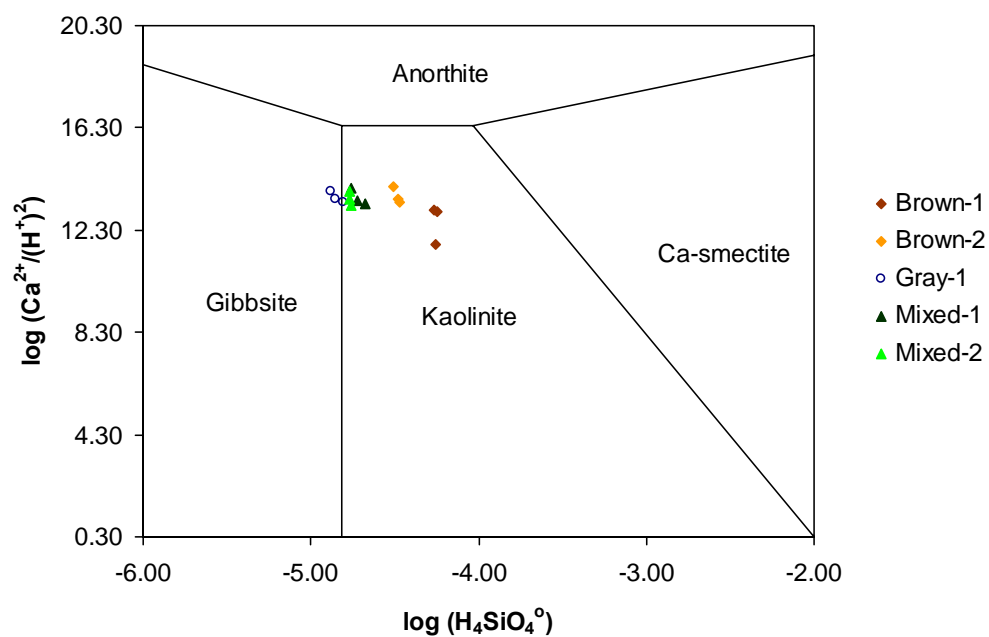


FIGURE 1.10. Stability field of Ca-Al silicate phases.

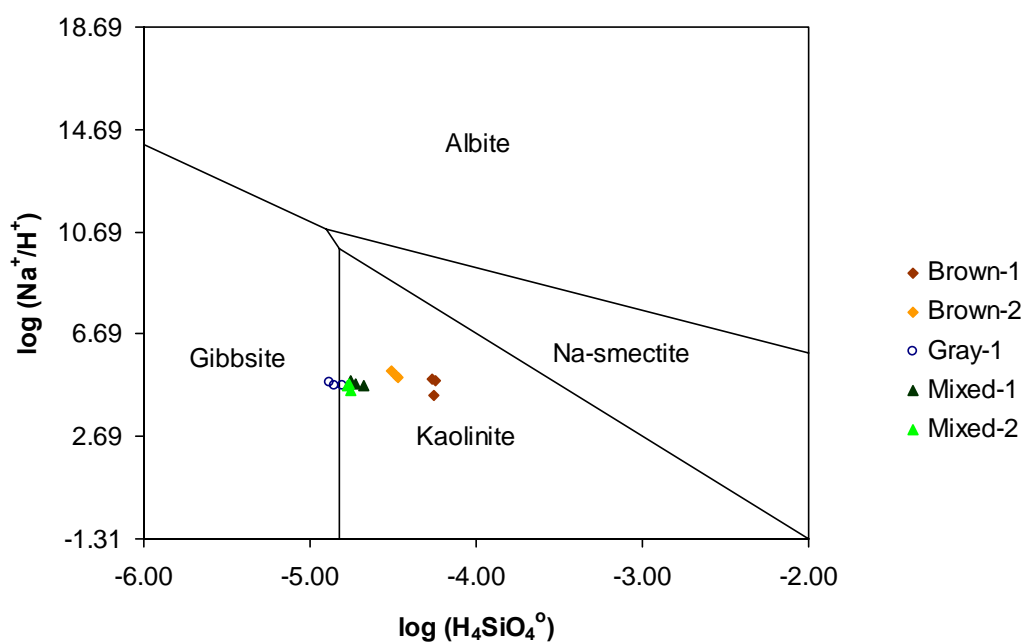


FIGURE 1.11. Stability field of Na-Al silicate phases.

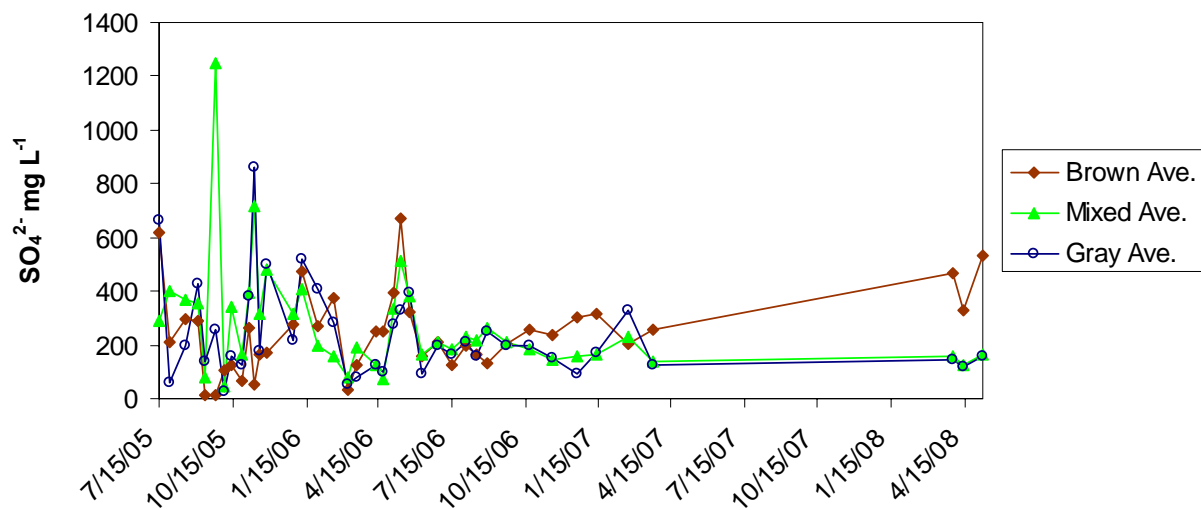


FIGURE 1.12. Sulfate levels averages for each plot, 2005-2008.

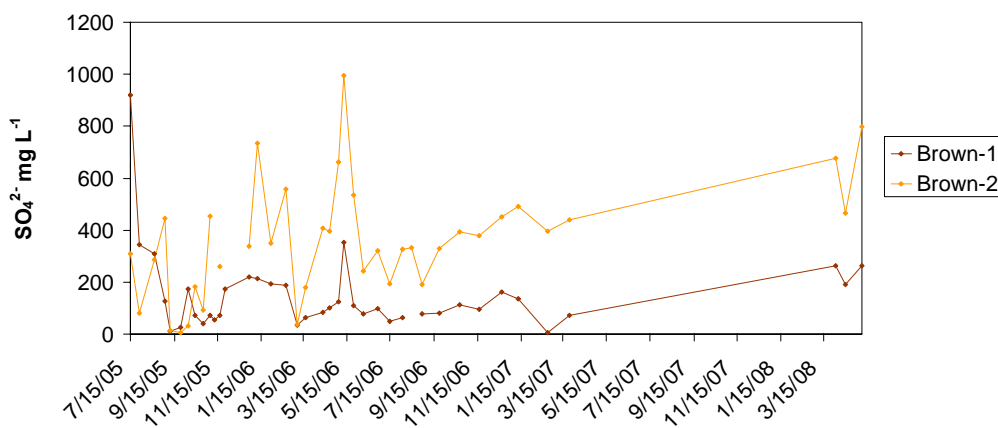


FIGURE 1.13. Sulfate levels for each brown sandstone plot, 2005-2008.

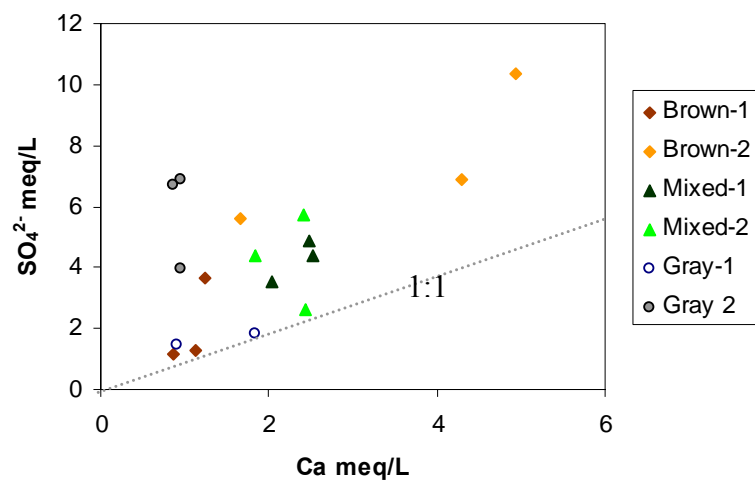


FIGURE 1.14. Scatter plot of Ca²⁺ vs. SO₄²⁻, May 2006 data

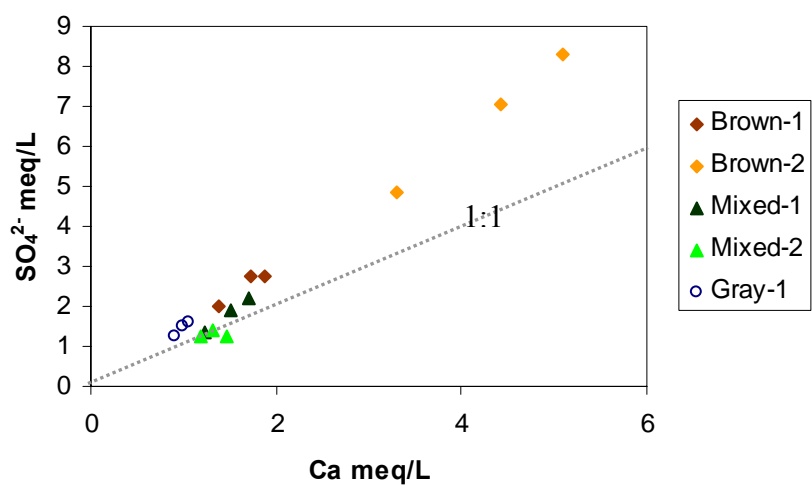


FIGURE 1.15. Scatter plot of Ca²⁺ vs. SO₄²⁻, April-May 2008 data.

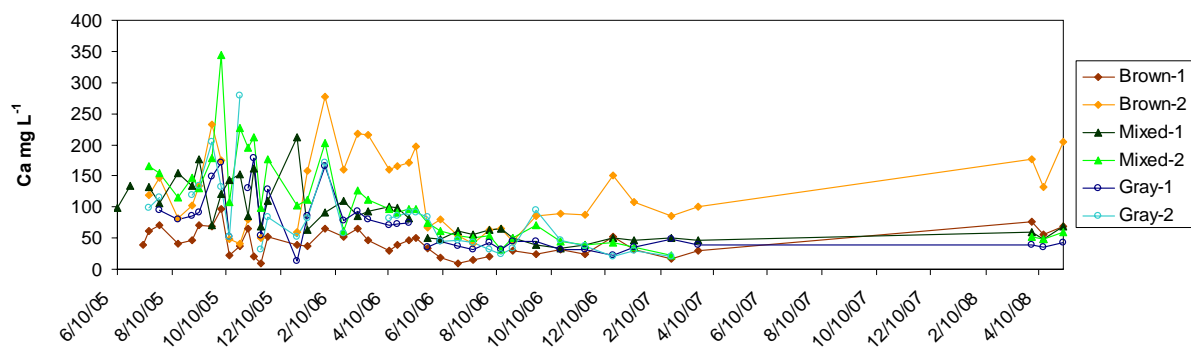


FIGURE 1.16. Calcium levels for all plots, 2005-2008.

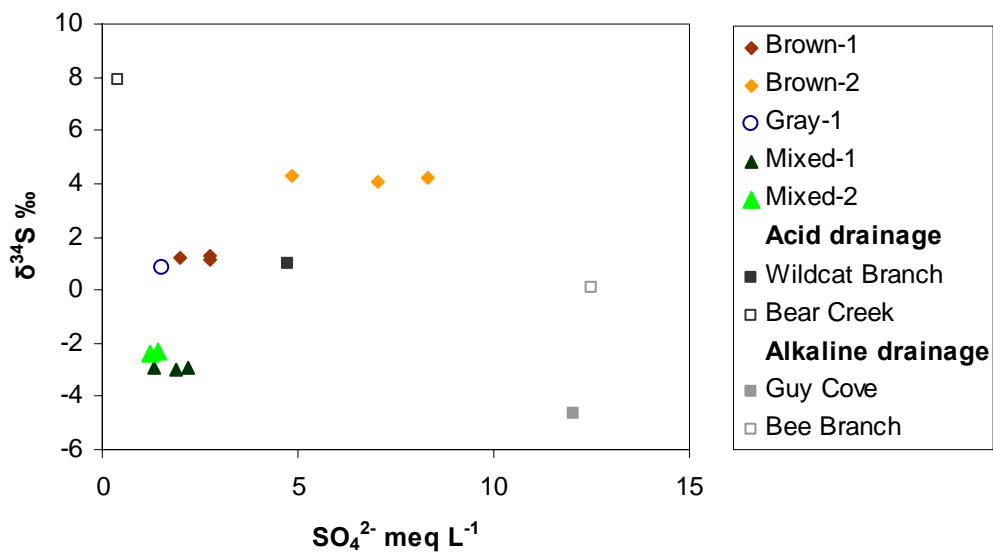


FIGURE 1.17. Scatter plot of $\delta^{34}\text{S}$ vs. SO_4^{2-} .

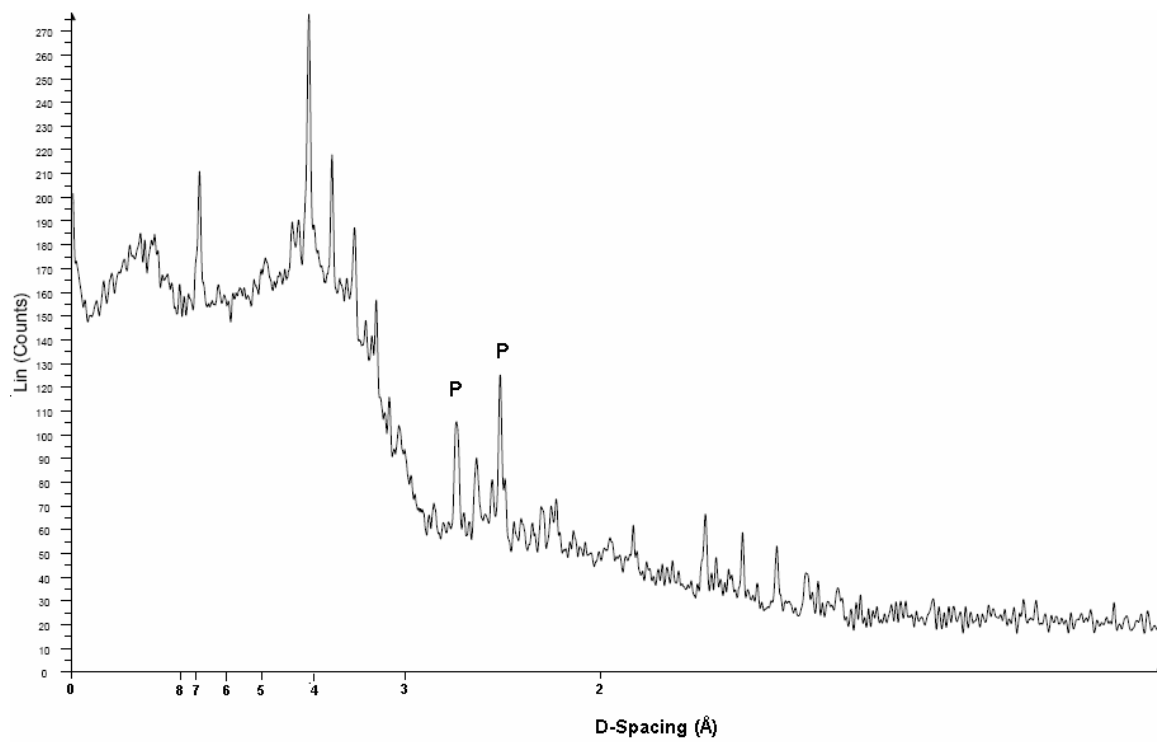


FIGURE 1.18. XRD analysis of coal from brown-2. “P” indicates pyrite peaks.

CHAPTER 2

OCCURRENCE OF *PHYTOPHTHORA* IN LOOSE-DUMPED MINE SPOILS AND INFILTRATED SPOIL WATERS

2.1 INTRODUCTION

The influence of geochemistry and spoil physical parameters on soil pathogens is not well known, but of interest with regard to forest health and reforestation success. *Phytophthora* are soil-borne plant pathogens that pose a significant threat to overstory and understory species and cause significant environmental degradation (Hardham, 2005). Common in eastern oak forests (Balci, Y. et al., 2007) and streams of eastern Kentucky (de Sá and Barton, 2007), *P. cinnamomi* and other *Phytophthora* species could have significant consequences toward afforestation efforts of American chestnut hybrids in the eastern U.S. (Rhoades et al., 2003; French et al., 2007, 2008). The occurrence of *Phytophthora* and site factors favoring increased aggressiveness of the pathogen have implications for tree performance. Mineralogy, water quality, and soil characteristics may influence its presence on the surface, as well as subsurface viability. Additionally, soil and water properties that affect tree nutrition and uptake may benefit or hinder the tree's response to disease caused by *Phytophthora*.

Phytophthora spp. may be introduced into a site by surface water, rain splash, wind, and the movement of soils and host species. Site factors that govern the presence and aggressiveness of *Phytophthora* to susceptible species include infiltration rate, elevation and climate. Rhoades et al. (2003) found that compacted soils with high moisture contents were more conducive to root rot caused by *P. cinnamomi*. High soil compaction levels from excessive grading are a common problem on surface mine lands in Appalachia (Burger et al., 2005; Angel et al. 2006, 2008; Sweigard et al., 2007a; Sweigard et al. 2007b). Compaction not only impedes forest establishment by restricting root growth, infiltration and aeration, but highly graded sites may be ideally suited for *Phytophthora* infection. Loose-graded spoils as described in the Forestry Reclamation Approach (FRA) (Burger et al., 2005; Sweigard et al. 2007a, 2007b), however, provide

conditions necessary for forest establishment and may be initially devoid of plant pathogens if the spoils are fresh and derived from subsoil geologic strata.

At the Bent Mountain, Kentucky, reforestation site, a long-term study is underway to determine the suitability of various mine spoils for reforestation efforts. These spoils include weathered brown sandstone, gray sandstone, shale, and mixed mine-run spoil, which differ in physiochemical properties and age of emplacement. In an effort to determine the presence of *Phytophthora* species, in particular, *P. cinnamomi*, on various spoil types reclaimed using FRA, soil and water samples were analyzed to detect their occurrence as influenced by spoil physiochemical properties and age of emplacement. In addition, infiltrated mine spoil waters were sampled to determine the possibility of *Phytophthora* transport in the subsurface and potential influence of aqueous geochemistry and spoil matrix properties on presence and viability of the pathogen.

2.1.1 THE GENUS *Phytophthora*

Phytophthora is a cosmopolitan genus with many species occurring in forests and natural ecosystems. Some species are major plant pathogens causing considerable degradation in forest environments, such as *Phytophthora cinnamomi* and Jarrah disease of Eucalyptus in Australia, and *P. ramorum* causing sudden oak death in the Pacific southwest United States. The most current taxonomy places the genus *Phytophthora* in the kingdom Chromista, phylum Oomycota, order Peronosporales and family Pythiaceae (Hardham, 2005). The genus *Phytophthora* was previously placed in the kingdom Mycetaceae with the true fungi, but it has been established that members of the Pythiaceae family are more closely related to brown algae, as evidenced by the presence of flagellate zoospores and cellulose rather than chitin as the major component of the cell wall, among other characteristics. The mycelium consists of aseptate hyphae with right-angled branches that are unpigmented in young cultures. Asexual reproduction occurs by formation of ovoid to lemoniform sporangia borne on sporangiophores similar to hyphae. Biflagellate zoospores are formed within the sporangium in water and are released through an apical pore. In the genus *Pythium* a vesicle containing cytoplasm flows from the sporangium, and zoospores are differentiated in the vesicle, and released into the surrounding water. This characteristic can be used to differentiate the two genera. The

zoospores are the major propagules for infection; they are motile and can swim for several hours, eventually forming a cyst that will germinate by producing a germ tube and mycelium. In sexual reproduction an oospore is formed by fertilization of an oogonium by a nucleus from an antheridium. Oospores are resting structures with thick walls that can remain in the soil for many years; for many species of *Phytophthora* these are the primary source of inoculum for new disease cycles. Chlamydospores are asexual globose structures with walls that may be thick or thin, and can also persist in the environment for varying amounts of time. The morphological characteristics and position of sporangia, oogonia, antheridia and chlamydospores are important for the identification of species. Presence or absence of hyphal swellings, and colony type on culture medium can be of value for preliminary identification of species but are insufficient for conclusive identification (Mitchell and Kannwischer-Mitchell, 1992; Erwin and Ribeiro, 1996).

Surveys for various *Phytophthora* species have been carried out in forested areas of the eastern U.S. on a limited scale. Based on a recent large-scale study on the prevalence of *Phytophthora* species in the eastern and central U.S. oak forests, the most commonly isolated species were *P. cinnamomi*, *P. citricola*, *P. europaea*, *P. cambivora*, *P. quercina*-like. Two previously unknown species were also isolated and are referred to as *Phytophthora* sp. 1 and *Phytophthora* sp. 2. These species may under-represent the full species assemblage as diverse types of vegetation were not surveyed (Balci, Y. et al., 2007). *P. cinnamomi* is among the most commonly isolated species in the eastern U.S. and has been identified as the cause of root rot on *Castanea dentata* (American chestnut) by Rhoades et al. (2003) and on *Quercus* spp. (oak) (Erwin and Ribeiro, 1996). *P. cinnamomi* may potentially cause disease on trees planted at Bent Mountain.

Some factors that may influence the survival and aggressiveness of various *Phytophthora* spp. on a particular host species include climate, soil temperature, soil pH, soil moisture, soil type, infiltration rate, topography, seasonal variation, and soil microbial communities. The most significant factors, especially for *P. cinnamomi*, appear to be associated with poorly-drained soils (for example, Rhoades et al. [2003]). Soil moisture is an important site factor governing the aggressiveness of *Phytophthora* to susceptible species. Rhoades et al. (2003) found that compacted soils with high moisture contents were more conducive to root rot caused by *P. cinnamomi*. The life cycle of *P.*

cinnamomi is dependent on saturated soil, as motile zoospores are only released in saturated soils. *P. cinnamomi* was isolated more frequently from soils with finer texture (clay >15% and silt >30% content) that are able to retain more water (Moreira and Martins, 2005). Although water is needed for propagation, *P. cinnamomi* may form resistant chlamydospores that persist in dry conditions for up to 10 months (Weste and Vithanage, 1979). Chlamydospores have been reported to survive in soil or roots from one to six years (Hwang and Ko, 1978; Zentmyer and Mircetich, 1966).

Soil matric potential has been used in previous studies to assess sporangium production (i.e. infection rate) of *Phytophthora* spp. in the soil environment. However, subsequent reflooding to achieve 0 mb is necessary to produce zoospores. *P. cinnamomi* is most active between 0 and -100 mb and causes little damage to root systems at -250 mb (Sterne et al., 1977a, b). The greatest percent of sporangium production (over 50% of total production) for *P. cinnamomi* is -40 to -300 mb (Gisi et al., 1980) and for *P. cryptogea*, between -10 and -3000 mb (Duniway, 1975a, b).

Phytophthora species are inhibited at varying temperatures. *P. cinnamomi* is inhibited where the average annual minimum temperature is below -20°C, and has not been detected in the U.S. beyond 40° N latitude where colder winters occur (Balci et al., 2007). Optimally, soil temperature for pathogenic activity by *P. cinnamomi* is 24-28°C (Swiecki and Bernhardt, 2006) but sporangium formation and zoospore release may occur at a temperature as low as 12 °C (Shew and Bensen, 1983).

Baiting success in isolation of *Phytophthora* species varies with time of year, with twice as many isolates obtained in spring versus fall (Balci et al., 2007). Seasonal and climate variation has been studied in three different forest ecosystems in Victoria, Australia, with regards to *P. cinnamomi*. Soil populations were highest in spring and summer but declined with lower rainfall. Populations decreased in winter when temperatures were under 10 °C (Weste and Ruppin, 1977). Higher amounts of rainfall have been noted as a factor strongly influencing the number of trees affected by *Phytophthora* dieback and collar rot on American chestnut hybrids (Griffin, 2008)

In several studies, it was shown that availability of soil nutrients, especially phosphorus, aided in survival of *P. cinnamomi* as there was less competition from other microbial communities (Shearer and Tippet, 1989; Moreira and Martins, 2005). As it is

not a competitive saprophyte, general suppression of *Phytophthora* occurs when microbial communities are diverse, as in nutrient rich soils (Sylvia et al., 2005). However, a minimal amount of organic matter and activity by other microorganisms is considered to be necessary for survival, as shown in a study of chlamydospore persistence in various soil conditions (Weste and Vithanage, 1979). The authors attributed this to the fact that exogenous nutrient supply by other microorganisms is needed for survival.

Soil pH influences the pH of infiltrated water and is a key component of bioavailability of soil minerals and compounds (Lindsay, 1979). The role of soil pH on *Phytophthora*-like organisms, such as the *Pythium* spp., has been shown to be highly significant on influencing the saprophytic activity and life cycle, but variable between species of the genus (Martin and Loper, 1999). In general, suppression of these organisms may occur at pH values from 6.6 to 8.0. No correlation was found between soil pH and the presence of *Phytophthora* in the Y. Balci et al. (2007) study, where soil pH values ranged from 3.7 to 7.4. More sampling is needed to better determine the influence of soil pH on *Phytophthora* in the soil.

Certain environmental factors may predispose plants to infection by *Phytophthora*. These factors induce stress on the host, possibly susceptibility to infection, and include drought, soil salinity, and waterlogged soils. Drought stress causes an uncertain physiological response in the plant that may render it more susceptible, especially when soils are flooded after a period of drought (Erwin and Ribeiro, 1996). However, in a study of *Eucalyptus marginata*, drought stress actually inhibited colonization by *P. cinnamomi* (Lucas, 2003). Trees stressed by flooded soils are more likely to be infected by *Phytophthora*, most likely due to the depletion of oxygen levels in soil (Erwin and Ribeiro, 1996). Salinity stress inhibits root defense systems in some species, as demonstrated by the addition of NaCl (0.1 and 0.2 M solutions) to soil (MacDonald, 1982, 1984).

Phytophthora species have been reported in many types of surface waters including irrigation canals, lakes and streams (Hwang et al., 2007), and *P. cinnamomi* is known to move by surface waters over long distances fairly rapidly (Kinal et al., 1993). *P. cinnamomi* also is spread by the movement of soil and infected organic matter (Hardy

et al., 2001). Current reforestation efforts at surface mines in Western Australia, where *P. cinnamomi* threatens Jarrah (*Eucalyptus marginata*) forests, demonstrate that transport of the pathogen to pathogen-free areas may be avoided by restricting movement of infested soil and water to these sites (Colquhoun and Kerp, 2007).

The transport and viability of *P. cinnamomi* and other *Phytophthora* in the subsurface, in particular, of zoospores, have been studied under a limited set of conditions. Movement in the subsurface by active taxis of zoospores towards favorable infection sites has been documented for various soils and water potentials. This movement occurs on a small scale, as zoospores can move towards infection sites 35 to 48 cm away when water potential is greater than $\Psi_m = -50$ mb and pore sizes are relatively large and continuous (Wilkinson et al., 1981). Zoospores move toward favorable infection sites likely in response to chemical gradients and may travel several centimeters at 200 $\mu\text{m/s}$ (Hardham, 2005). Kinal et al. (1993) studied passive transport of *P. cinnamomi* in a lateritic soil profile in Western Australia, and measured the movement of inoculum by subsurface perched water flowing laterally. The soil profile consisted of 0-1 m of gravelly sand over a lateritic duricrust 1-2.5 m thick, which overlies kaolinite clay. The lateral, rapid dispersal of propagules by water over relatively long distances occurs when pore size is larger than propagule size, the water flows laterally, and the pore connections are continuous. When the lateritic duricrust is removed during mining for bauxite and the area is restored, water ponding does not occur and infiltration rates are greater. Therefore, even when *P. cinnamomi* is present, Jarrah trees demonstrate high survival rates (Koch and Samson, 2007).

Species arrival and diversity at disturbed sites has been studied at coal mines in Europe and Wyoming (Wanner and Dunger, 2002; Anderson et al., 2008), and other reclaimed mined areas are of interest in studies of ecological succession. The possible role of *Phytophthora* as an early colonizer of disturbed areas is not known. This project has the potential to provide a chronosequential key to the arrival of *Phytophthora* species as loose-dumped mine spoils 1 and 3 years old were tested. The soil mixed in with the brown sandstone spoil, and in lesser amounts in the mixed spoil, is host to microbial populations, whereas the gray spoil has no soil content and should be mostly devoid of soil microorganisms when emplaced.

2.1.2 AMERICAN CHESTNUT

American chestnut (*Castanea dentata* (Marsh.) Borkh.) was a dominant overstory species in eastern forests until nearly devastated by the arrival of the exotic fungus *Cryphonectria parasitica* (Murr.) Barr, cause of chestnut blight, in the early 20th century. Over 4 billion trees were destroyed by 1950 and the species was reduced to an understory tree now classified as endangered (Hepting, 1974). In eastern U.S forests, sprouts from blight-killed trees often do not often reach reproductive maturity (Russell, 1987).

The American Chestnut Foundation has bred American chestnut hybrids for resistance to chestnut blight by crossing resistant pure American chestnut with Chinese chestnut, *Castanea mollissima* (Blume). The goal is to have a tree that is phenotypically identical to the original species (Hebard, 2006): mostly American chestnut in character with added blight resistance from the Chinese chestnut.

In addition to studying site characteristics from the natural range of American chestnut, silvicultural studies are underway to aid restoration efforts. Although these findings indicate that American chestnut was fairly well-suited to diverse conditions in its natural range, challenges exist for reforestation and afforestation efforts (Jacobs, 2007). *Phytophthora* species such as *P. cinnamomi*, *P. cactorum*, and possibly others have caused high mortality to hybrid strains at experimental plantings at forest sites and orchards (Brosi, 2001; Rhoades et al., 2003; Griffin, 2008). However, careful site selection by avoiding poorly drained soils that increase susceptibility to the pathogen may reduce or eliminate the threat of *P. cinnamomi* and other *Phytophthora* species (Sisco, 2007). Incidentally, the area of coal mining in Appalachia correlates well with the natural range of American chestnut; reclaimed mine sites present a location where American chestnut may thrive. Reclaimed mine sites in Eastern Kentucky have been proposed as a place of reintroduction for the American chestnut as they are well-drained and potentially devoid of *Phytophthora*. Currently, loose-dumped mine spoils at the Bent Mountain site are under investigation regarding the suitability for American chestnut (French et al., 2008).

2.2 OBJECTIVE

The subjects investigated in this chapter, with regards to forest establishment on different types and ages of mine spoils, include the presence of *Phytophthora* and the potential for subsurface transport of *Phytophthora* through the spoils, as influenced by infiltrated water geochemistry. Variations in spoil geochemistry and physical properties, whether due to initial spoil geochemistry or weathering processes, may inhibit or prohibit the presence and subsurface transport of *Phytophthora*. Alternatively, certain spoil geochemical and physical properties may predispose trees to infection by *Phytophthora* by inducing stress.

2.3 METHODS

2.3.1 STUDY SITE

In addition to the 2005 plots (Figure 2.1) discussed in the previous chapter, 12 additional half-acre (0.2-ha) research plots consisting of end-dumped gray sandstone, brown sandstone, shale, and mixed sandstones and shale (three plots of each) were constructed in March 2007 (Figure 2.2). These plots will henceforth be referred to as 1-year plots whereas the 2005 plots will be referred to as 3-year plots. All plots were planted with native hardwood species, including: white ash (*Fraxinus americana*), white oak (*Quercus alba*), northern red oak (*Quercus rubra*), chestnut oak (*Quercus prinus*), sugar maple (*Acer saccharum*), American sycamore (*Plantanus occidentalis*), white pine (*Pinus strobus*), black locust (*Robinia pseudoacacia*), dogwood (*Cornus florida*), redbud (*Cercis canadensis*) and a blight resistant hybrid of American chestnut at a density of 1,200 trees per hectare. These plots were used as chronosequential sites for surface sampling of *Phytophthora* species.

2.3.2. SOIL AND WATER CHEMISTRY

Composite soil samples were taken from the 3-year plots in July of 2005 and 2007 in a systematic random technique. The plots were equally divided into sixteen subplots, eight of which were randomly selected. From these, five sub-samples were taken with a sampling spade to a depth of approximately 3 cm from each subplot and mixed together

to make a composite sample. Five soil samples each from Berea and Robinson forests were collected at 0-15 cm depth. The soil samples were dried at 50° C for 1 week.

A micropipette method (Miller and Miller, 1987) was used to analyze for sand, silt, clay, and textural class. Soil samples were analyzed at the UK College of Agriculture Regulatory Services Laboratory. A combination of 10 g of soil and 10 mL of water was used to measure soil pH; to the slurry, 10 mL of Sikora buffer was added to measure buffer pH (Sikora, 2006). The ammonium acetate method (Summer and Miller, 1996) was used to assess cation exchange capacity (CED) and exchangeable bases.

Cations and phosphorus were extracted by the Mehlich III method (Mehlich, 1984). Total carbon and nitrogen were analyzed by dry combustion by the Kentucky Agricultural Experiment Station, Lexington, Kentucky.

Soil moisture and soil temperature probes were installed on the 3 year plots in July 2006. Data were recorded hourly at depths of 50, 125, and 200 cm by probes connected to a Campbell Scientific CS10X datalogger.

Water geochemistry was analyzed for waters infiltrating the 3-year plots. Field measurements were taken for pH, temperature, and electrical conductivity (EC).

2.3.3 SOIL BAITING

A soil baiting method was adapted for detection of *Phytophthora* in soils from the 3-year and 1-year plots at Bent Mountain. Soil baiting occurred monthly from May to October 2007 and also in May 2008. Five sub-samples of mine soil from a depth of up to 10 cm were composited from each site to create approximately 1 kg of sample. Sampling equipment (trowels, hands, tubs, etc.) were sterilized with a 10% bleach solution and thoroughly rinsed with deionized water between each sampling site, but not between sub-samples. The soil baiting procedure was adapted from previous methods to accommodate a larger, composite soil sample (Erwin and Ribeiro, 1996; Tsao, 1983). *Rhododendron maximum* leaves were collected the same day as the spoil was sampled and from the same site for each round of sampling. Leaves were cut from the branches, placed in Ziploc[®] bags and stored in a cooler on ice until ready for baiting (within one day of collection). Each soil sample was weighed out to 1 kg and placed into a sterilized, thoroughly rinsed (10% bleach; deionized H₂O) 2 gal. (7.57 liter) plastic tub.

Enough distilled water was added to the tubs to cover the soil to a depth of about 2 inches (5 cm). Rhododendron leaves were cut with a sterile razor into pieces of approximately 1 cm² and 12 pieces were floated on the surface of the water in each tub. The tubs were covered with foil and left at room temperature for 72 hours. Leaf pieces were removed from the immersed soil and inspected for symptoms of *Phytophthora* infection, i.e., brownish-black discoloration and water soaking. These sections were excised and placed into PARPH-V8 agar plates (Jeffers and Martin, 1986; Ferguson and Jeffers, 1999) and incubated at 25 °C for 1 week. PARPH-V8 is a selective growth medium for most *Phytophthora* species (see Appendix A). Colonies growing on the PARPH-V8 agar plates were isolated and transferred to new PARPH-V8 agar plates; two transfers were made to ensure pure cultures.

The baiting included a positive control for *Phytophthora* using soils from Robinson and Berea forests (Figure 2.3), where *P. cinnamomi* had previously been detected. A tub of deionized water and rhododendron leaf pieces covered with foil served as negative control (Figure 2.4). In addition, excisions from leaves collected from the site were plated directly in agar each month to ensure that the rhododendron was free of *Phytophthora* infection at the time of collection.

2.3.4 WATER FILTERING

Waters infiltrating through the plots were tested three times during the sampling period. During 2007, drought limited flow of the waters, and water was typically collected within days of a rain event when drainage was present. Water was collected in 1-L bottles directly from each of the six monitoring stations during July and November 2007, and May 2008 and stored in a cooler. The monitoring stations drain by means of lysimeters and PVC pipes. Three 4.6-meter-square lysimeters in each plot that are drained to the exit points by PVC pipes nominally 2.5 centimeters in diameter. Each plot is isolated from the others by a 2.5-meter buffer zone and drains into its own sample monitoring station. During July 2007 and May 2008, water was not collected for the 2005 gray-2 plot as no water was flowing. Water samples were filtered in the lab in 100-mL aliquots through a 3-µm polycarbonate filter (Whatman Nuclepore Track-Etch Membrane), which was sized to capture *Phytophthora* spp. propagules, i.e. sporangia,

zoospores, chlamydospores, hyphal fragments, and colonized organic matter. Each filter was placed face down on selective medium (PARPH-V8) plates and incubated at 25 °C in the dark. Filters were removed after one week and individual colonies were transferred into new medium.

Water samples were collected during July 2007 and May 2008 from Clemons Fork in Robinson Forest, from which *P. cinnamomi* previously had been isolated using rhododendron leaves as baits (de Sá et al., 2007).

For samples collected in November 2007 and May 2008, 100 - 500 mL aliquots were filtered through a 3-µm polycarbonate filter (Whatman Nuclepore Track-Etch Membrane); the quantity of water filtered was dependent on turbidity. The brown sandstone waters had higher turbidity. The brown-1 water was filtered in two 100-mL aliquots, and the brown-2 water was filtered in two 250-mL aliquots. Filters were placed in sterile containers and stored at -20°C until DNA extractions were performed. DNA was extracted from the filtrates using an UltraClean™ Water DNA Isolation Kit (Mo Bio Laboratories) and methods provided by the manufacturer. PCR amplifications of total DNA were performed using two pairs of primers amplifying rDNA as described below.

2.3.5 ISOLATE IDENTIFICATION BY MORPHOLOGY

Colony morphology of isolates obtained from soil baiting and water filtering was observed for mycelium habit and pattern. Isolates obtained from soil baiting and water filtering with morphology characteristic of *Phytophthora* were transferred twice and isolates with *Pythium* growth patterns (i.e. rapid mycelial growth and fine flexuous hyphae) were discarded. Colonies underwent a sporangium production test (Jeffers, 2006) using a non-sterile soil extract solution (1.5%) (Jeffers and Aldwinckle, 1987). Colony morphology of isolates was observed for mycelium habit and pattern (Figures 2.5-2.8). At 200x magnification, structures present in the agar, such as hyphal swellings, chlamydospores, and sporangia, were observed and used for a preliminary identification of *Phytophthora* (Figures 2.9-2.11). Forty-one second-transfer isolates obtained from soil baiting and water filtering showed some structures characteristic of *Phytophthora* at 200x magnification and were selected for identification using a polymerase chain reaction

(PCR) based molecular approach, by amplification of ribosomal DNA (rDNA), followed by sequencing of amplified fragments.

2.3.6 MOLECULAR IDENTIFICATION OF *Phytophthora* ISOLATES

Mycelium was scraped from the surface of culture plates and approximately 200 µg were used for DNA extraction with the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) according to manufacturer's instructions. The purified DNA was used in PCR amplifications of internal transcribed spacer regions (ITS) using the primers ITS 6 and ITS 4 (Cooke et al., 2000) (Table 2.1). *P. cinnamomi* specific primers CIN 3A – CIN ITS 4 (Anderson, 2006) (Table 2.1) were also used for PCR amplifications of DNA from some of the isolates in separate reactions. PCR amplification reactions contained 25 µL GoTaq® Green Master Mix (Promega), 2 µl of the DNA template diluted 1/50, 2.5 µL of each primer, and 18 µl molecular grade water. PCR cycling parameters were carried out according to Anderson (2006) and consisted of a 5-minute denaturation at 94 °C followed by 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 74 °C for 1 minute, and a final extension of 74 °C for 5 minutes. PCR products were visualized by electrophoresis in 0.7% agarose gels stained with ethidium bromide.

DNA from PCR products was quantified for sequencing using the SybrGold stain on a FluorImager 595, and direct sequencing of the PCR products was performed by the Sanger reaction using the ABI Big Dye terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3730xl DNA Analyzer at the University of Kentucky Advanced Genetics Technology Center (UK-AGTC). The primer pairs ITS 6 - ITS 4 and CIN 3A - CIN ITS 4 were used to sequence PCR products in the forward and reverse direction. DNA sequences were compiled, edited and analyzed using ChromasPro (Technelysium Pty. Ltd, Tewantin, Australia) and sequence identity searches were performed using BLAST (www.ncbi.nlm.nih.gov). Sequences were deposited in GenBank.

2.4 RESULTS

2.4.1. SOIL AND WATER GEOCHEMISTRY

Although this was not necessarily a comparative study of forest soils and reclaimed mine soils, some comparisons can be made between sites where *Phytophthora* was detected (the forest sites and the 3-year brown sandstone) versus the rest of the mine spoils. These analyses may be useful in delineating soil properties that favor the presence of *Phytophthora*.

Soil geochemistry varied between mine spoil types, and between the mine spoils and forest sites (Tables 2.2-2.4). Notably, the soil pH values of brown spoil (6.6 to 6.7) and of soils from Berea and Robinson forests (5.5-5.6) were both moderately acidic, whereas gray and mixed spoils greater had pH values of 8.

Phosphorus values for the brown sandstone were higher than for other spoil types or forest sites; this nutrient decreased from 1-year to 3-year plots. Phosphorus values of forest sites were intermediate between those of mixed, gray, or shale mine spoils and brown sandstone.

Soil temperature was comparable for all spoil types during the study period. Soil moisture was highest for brown sandstone spoil during the 2007 study period, approximately 20% to 30% moisture compared to 10% moisture in the mixed and gray plots (Table 2.5, Figure 2.12).

Infiltrated water chemistry results for May 2008 waters are presented in Tables 2.6a and b. During this sampling event, pH and temperature were comparable for all plots; the mixed plots had a much greater EC value. Brown spoils, in comparison with other spoil types, were higher in silica (Si), chlorine (Cl⁻) and sodium (Na⁺).

2.4.2 DETECTION OF *Phytophthora* IN SOIL SAMPLES

Phytophthora spp. were consistently isolated from forest soils using the leaf baiting method judging from characteristics of the cultures on PARPH-V8 plates and from the morphology of the isolates. PCR amplification products were seen on agarose gels when ITS 6 and ITS 4 primers and *P. cinnamomi* specific primers were used. Sequence analysis of 46 PCR products from 41 samples indicated that *P. cinnamomi* was

successfully baited from soils from Berea and Robinson forests. It was possible to amplify the ITS region of *P. cinnamomi* using the ITS 6 and ITS 4 primers from isolates obtained from seven forest soil samples, and two to four sequences were used to generate a consensus sequence for each sample. BLAST searches of consensus sequences for each sample indicated 100% identity with the sequence of multiple isolates of *P. cinnamomi*, and sequences with GenBank Accession numbers AY302143.1 and AY302147 were chosen to make the alignments in Figure 2.13. Three of the samples from forest soils that yielded ITS sequences with high percentage of identity to *P. cinnamomi* were confirmed by PCR amplification of total DNA with *P. cinnamomi* specific primers CIN 3A and CIN ITS 4 (Figures 2.14 and 2.15). For one isolate recovered from soil from Robinson Forest the sequence obtained from PCR amplification fragments using ITS6 and ITS4 primers had 94% identity with *Pythium macrosporum* (accession numbers: AB359910 and AF452145).

P. cryptogea was amplified from a sample from the brown-2 plot. No other mine spoil yielded isolates with sequence identity to *Phytophthora* species (Tables 2.7 and 2.8). Amplification of total DNA using ITS 6 and ITS 4 primers from seven isolates obtained by baiting mine spoils resulted in sequences with high identity to *Mortierella* spp. Four other isolates yielded sequences with high percentage of identity to sequences of unidentified fungi. *Mortierella* is a common soil zygomycete that can sometimes occur as a contaminant in isolation plates. Only three of the 41 sequenced samples yielded poor sequences. See Table 2.10 for isolate descriptions.

2.4.3 DETECTION OF *Phytophthora* IN WATER SAMPLES

The direct plating of the filters on the surface of the plates for transfer of trapped propagules resulted in a larger number of colonies on PARPH-V8 plates. Growth of colonies was observed from filters of all spoil types, but the majority of these colonies did not grow after being transfer to new PARPH-V8 plates. Of the isolates recovered on the second plates, only isolates from brown sandstone showed growth characteristics similar to *Phytophthora* (Table 2.9). Second transfer isolate data are reported in Table 2.10. *Phytophthora cryptogea* was isolated and sequenced from waters infiltrating the Brown 1 plot during May 2008. This species was also detected from the surface mine

spoil of the Brown 2 plot in the same sampling interval. *Pythium undulata* was recovered from water collected from Clemens Fork but not *P. cinnamomi*.

Due to several factors, successful amplification of DNA extracts from filtrate samples that had tested positive for *Phytophthora cryptogea* by the water filtering method was not achieved. The issue evokes the need to test DNA filter extracts with a variety of robust primers specific to different *Phytophthora* species, including *P. cryptogea*. Of the primers used, the ITS6-ITS4 set was too general, amplifying a variety of fungi and pythaceous species, and not robust enough to work in the presence of inhibitive chemicals; this primer is intended for use on pure cultures. The other primer set, CIN 3A-CIN ITS4, was specific for *P. cinnamomi* and fairly robust, but it was too specific, only amplifying two-thirds of *P. cinnamomi* isolate DNA from previous experiments. Additionally, a larger set of positive control filtrates concurrently tested by water filtering must be used to validate the method. Positive controls were not successfully obtained for *P. cinnamomi* by water filtering of Clemens Fork. Other impediments to DNA amplification may have included poor quality of the DNA extract and inhibitive chemicals. DNA may have degraded during the extraction process or during storage of filtrate at -20°C prior to extraction. Inhibitors to PCR amplification co-extracted with DNA from the filtrate may have acted to impede PCR reactions.

2.5 DISCUSSION

Phytophthora cinnamomi was consistently isolated from soils from Berea and Robinson forest using rhododendron leaves as baits. *Phytophthora cryptogea* was isolated in 2008 from the surface and infiltrated waters of the 3-year brown sandstone plots. No *Phytophthora* isolates were recovered from the 1-year brown sandstone plots, or from other types and ages of plots. Brown sandstone spoil was derived from surface and near-surface strata and had the greatest soil content as evident by woody debris and roots (Angel et al., 2006). Mixed spoil contains some brown sandstone spoil. Gray sandstone and shale spoil were derived from deeper, unexposed strata and therefore have no soil content. *Phytophthora* species are generally transported into new areas through movement of infested soil, plants and organic materials, mostly by human activity and also by animals, and in water or by air currents. It is likely that *Phytophthora* propagules

were present in the soil; longer exposure to the environment may have allowed for entry into the area. After weathering for 3 years spoil chemistry or nutrient availability may have become more favorable for retention of propagules in the spoil and growth of *Phytophthora* in organic matter and plants to reach detection levels. Inherent qualities of the brown sandstone spoil, including pH and moisture, may be more conducive to the survival and viability of *Phytophthora* spp. such as *P. cryptogea* as compared to the other spoil types. Soil pH for both brown plots averaged about 6.5 compared to 8.3 in the mixed and gray plots. Soil sampling for *Phytophthora* in the eastern U.S. has recently been conducted on soils ranging from 3.7 to 7.4; no correlation between *Phytophthora* and pH was observed, but soils at higher pH's were not tested. At a pH of 8.3, nutrient uptake may be inhibited.

Numerous studies have shown that increased soil moisture increases survival and release of zoospores by various *Phytophthora* species. Greater soil moisture of the brown spoil is due to a greater amount of clay particles that absorb water and decrease pore space (Angel, 2008).

The presence of greater ground cover, more colonizing plant species and a greater amount of organic matter are likely to have been important in the survival of *Phytophthora* propagules, leading to growth and detectable levels of the pathogen in the 3-year brown plots. Although none of the colonizing plant species have been reported as hosts of *Phytophthora*, it is possible that a host species was present. Due to higher soil moisture resulting from higher precipitation, and the presence of susceptible plants and organic matter, pathogen density may have increased sufficiently for detection.

A related aspect could be the presence of greater numbers of colonizing microbial communities in the brown spoil. Microbial activity may provide an exogenous nutrient supply for *Phytophthora* spp that may be necessary for survival, although *Phytophthora* spp. in general are poor saprophytes. The other spoil types were unweathered and probably did not supply the conditions necessary for survival of propagules.

Empirical methods were not employed to evaluate differences in soil microbial activity between spoil types. It is probable that initial soil content and time since emplacement promoted more activity by soil microfauna in 3-year brown sandstone as compared to other plots. Conversely, lower soil moisture, higher soil pH, and less activity

by soil microfauna in gray and mixed spoil may be detrimental to growth and survival of propagules of *Phytophthora* species.

After one month, as the inhibitory effects of the PARPH-V8 selective medium decreased, *Pythium* spp. and *Mortierella* spp. grew in the recovery plates from every plot type except for the 2007 shale plots. The presence of these microbial communities, which was confirmed by PCR, may be the result of contamination during any step of the baiting process, or indicate that they could be of significance as colonizers of newly reclaimed mine spoils. However, as these zygomycetes do not pose a great threat to hardwood trees, the majority were not analyzed by DNA methods in this study. *Pythium* spp. have been shown to grow faster than certain *Phytophthora* spp in agar, thus the *Phytophthora* is not detected (Erwin and Ribiero 1996).

The detection of *P. cryptogea* in infiltrated mine waters indicates that application of the filtering method for detection of *Phytophthora* from mine spoils may be successful and warrants further research. It may also be significant as passive, subsurface transport of the pathogen through 2-3 meters of matrix has not been documented. Infiltrated waters represent a larger sample of the entire plot rather than the top 10 cm of mine spoil. The filtration method had been proven more effective and efficient for the detection of *Phytophthora* species in forest streams than baiting methods. It also provided quantitative data on inoculum density when used for detection of *P. ramorum* and other species of *Phytophthora* in streams and natural ecosystems by Hwang et al. (2007).

Despite differences in soil texture and soil moisture, infiltration rates on all 3-year plots were comparable and high due to the presence of megapores and macropores within mine spoils. Taylor (2008) measured rainfall events from 2005 to 2006 and noted that discharge volumes (compared to amount of rainfall) averaged 11% and discharge duration ranged from 5 to 8 days. These conditions should be beneficial for establishment of trees as root systems of trees are less susceptible to *Phytophthora* infection in well-drained soils versus saturated, compacted soils.

The risk of *P. cryptogea* to trees growing at the Bent Mountain site is unknown given the species composition and site factors. The susceptibility of hybrid American chestnut to *P. cryptogea* is not certain, but some species of *Phytophthora* may pose a risk to these trees (Griffin, 2008). *P. cryptogea* has been documented in natural waters and

irrigation systems in the U.S., and it has a wide range of host species. It has been detected and identified as a cause of diseased Noble fir (*Abies procera*) seedlings in Ireland (Shafizadeh and Kavanagh, 2005) and of sugar pine trees (*Pinus lambertiana*) in an orchard in the western U.S. (USDA Forest Service, 2005). This species is commonly isolated in nurseries, where it causes root rot of ornamental woody shrubs (Ferguson and Jeffers, 1999). It is one of several species of *Phytophthora* that cause “Jarrah dieback” in southwest Western Australia (Shearer et al., 1987).

Field evidence for disease caused by *Phytophthora* was negative on all plot types. No host species of *Phytophthora* spp. at Bent Mountain (i.e. red oak, white oak, and American chestnut) showed symptoms of root rot or dieback caused by *Phytophthora*. No volunteer species at Bent Mountain that are hosts to commonly occurring *Phytophthora* spp. have been reported and no tree mortality due to *Phytophthora* has been observed (Angel, 2008; French et al., 2007).

The outlook for planting American chestnut hybrids at the site is promising as *P. cinnamomi* was not detected. Current reforestation efforts at surface mines in Western Australia, where *P. cinnamomi* threatens Jarrah (*Eucalyptus marginata*) forests, demonstrate that transport of the pathogen to pathogen-free areas may be avoided by restricting movement of infested soil and water to these sites (Colquhoun and Kerp, 2007). The pathogen is transferred mainly by soil and surface water and the Bent Mountain research site does not have hydrologic surface or subsurface inputs other than rain water.

PCR-based methods are considered to be among the most sensitive, specific and rapid methods for identification of *Phytophthora* spp. (Martin et al., 2004). Amplification of internal transcribed spacer (ITS) regions of rDNA are particularly useful because the rate of accumulation of mutations in these regions approximates the rate of speciation (Cooke et al., 2000). Sequence analysis of ITS regions has been widely used in identification of genera and species (Cooke et al., 2000; Martin et al., 2004; Villa et al., 2006; Balci, Y. et al., 2007). Furthermore, thousands of *Phytophthora* sequences have been deposited in GenBank and data search and analysis tools such as BLAST are available.

2.6 CONCLUSIONS

The objectives of this study were to analyze the occurrence of *Phytophthora* spp. in different types of loose-dumped mine spoils emplaced 1 and 3 years ago as well as in waters infiltrating the 3-year-old plots. Monthly testing of different types and ages of reforested mine spoils and infiltrated spoil waters in eastern Kentucky as well as forest soils for *Phytophthora* species was carried out monthly from May to October 2007 and in May 2008. *Phytophthora cryptogea*, as identified by sequence analysis, was recovered from the soil surface and infiltrated waters of the 3-year-old brown sandstone. Greater soil moisture, greater soil nutrient availability, lower soil pH and the presence of ground cover plants likely favored the presence of the pathogen compared to other plot types. Because these sites are well drained it is expected that trees growing in loose-dumped mine spoils emplaced by the FRA will have less mortality due to infection by *Phytophthora* species. Thus far, hybrid American chestnut on loose-dumped mine spoils has fared better than those growing in compacted or poorly-drained soils. Factors inherent to loose-dumped mine spoil (macropores and megapores) that result in high infiltration rates may decrease the occurrence of root rot diseases. Trees growing in well-drained soils at mine restoration sites in Western Australia where *P. cinnamomi* is present have shown less susceptibility to the pathogen than trees at natural forest sites where a hardpan layer of soil is present (Koch and Samson, 2007).

Monitoring of the establishment of trees on loose-dumped mine spoils, in particular, hybrid American chestnut, is needed over an extended period of time to examine growth, competition, and the influence of stress factors with regards to presence of *Phytophthora* species in the various mine spoils. Further research into the applicability of filtering and baiting methods to detect and monitor *Phytophthora* species is needed. The results indicated that it is possible using the baiting method to detect *Phytophthora cinnamomi* in forest areas, and it was possible to detect *P. cryptogea* using filtering. Disease caused by *P. cryptogea* on the brown plots was not observed, nevertheless, further work is needed to document the susceptibility of hybrid American chestnut to the pathogen. Sampling for *Phytophthora* spp. during years with greater precipitation may result in greater success in isolating them. Over a longer period of time, the influence of

rainfall variation, infiltration rate, soil microbial activity, and tree establishment with regards to *Phytophthora* occurrence should be evaluated.

TABLE 2.1. Sequence of primers used for PCR amplification of DNA and sequencing of PCR products of *Phytophthora* spp. isolated by soil baiting and water filtering.

PRIMER	PRIMER SEQUENCE	USES
ITS 6	5'- GAA GGT GAA GTC GTA ACA AGG - 3'	<i>Phytophthora</i> , other pythacious spp., some fungi
ITS 4	5'- TCC TCC GCT TAT TGA TAT GC – 3'	
CIN 3A	5' CAT TAG TTG GGG GCC TGC 3'	<i>P. cinnamomi</i>
CIN ITS 4	5' TGC CAC CAC AAG CAC ACA 3'	

TABLE 2.2. Properties of 1-year spoils. Standard errors in parenthesis.

SPOIL TYPE	pH	P	Ca ²⁺	Mg ²⁺	K ⁺	N	C	SAND	SILT	CLAY
			μg/g				Mg/g		%	
BROWN	6.7	11.5	409.8	147	32.8	0.2	3	80.8	17.1	2.1
	(0.1)	(0.5)	(15.7)	(22.4)	(3.1)	(<0.1)	(1)	(1.2)	(2.8)	(1.8)
GRAY	8.6	3.2	1151.6	376	54.5	0.1	11.6	79.1	18.1	2.8
	(0.2)	(1)	(296.3)	(49.3)	(4)	(<0.1)	(1.7)	(1.3)	(1.6)	(1.1)
MIXED	8.1	2.3	1604.3	322.3	61.6	0.3	15.3	79.3	17.5	3.2
	(0.2)	(0.6)	(471.1)	(35.6)	(6.9)	(<0.1)	(2.1)	(7.5)	(7.0)	(1.5)
SHALE	6.9	3.8	785.1	396.8	140.5	1.1	57.6	61.2	30.2	8.6
	(0.2)	(1.1)	(87.9)	(61.8)	(25.8)	(0.1)	(22.7)	(9.2)	(5.0)	(6.9)

TABLE 2.3. Properties of 3-year spoils.

SPOIL TYPE	pH	P	Ca ²⁺	Mg ²⁺	K ⁺	N	C	SAND	SILT	CLAY
			μg/g				mg/g		%	
BROWN	6.6	7.5	499.9	315.8	63.3	BDL	8.9	64.8	26	9.2
	(0.2)	(0.4)	(24.1)	(34.8)	(3.5)		(2.7)			
GRAY	8.6	2.5	941.1	336	62.4	BDL	6.3	73.6	18.9	7.5
	(0.1)	(0.7)	(92.1)	(24.7)	(3.5)		(0.9)			
MIXED	8.5	1.6	1306.8	288.4	68.7	BDL	7.6	67.9	24.1	8
	(0.1)	(0.1)	(82.3)	(7.5)	(4.6)		(1.3)			

TABLE 2.4. Properties of eastern Kentucky chestnut planting sites. Bear Mountain, Berea College Forest 37° 32' 19"N, 84° 14' 51" W, 335 m.a.s.l.; University of Kentucky Robinson Forest, 37° 28' 9"N, 83° 8' 3" W, 427 m.a.s.l.

SITE	pH	P	Ca ²⁺	Mg ²⁺	K ⁺	N	C	SAND	SILT	CLAY
			μg/g				mg/g		%	
BEAR MOUNTAIN	5.6	4.1	1252.4	230.5	93.2	1.9	22.4	10.8	71.2	18.0
	(0.2)	(0.5)	(180.1)	(19.7)	(10.5)	(0.2)	(2.7)	(2.3)	(2.2)	(2.0)
ROBINSON FOREST	5.5	5.6	909.8	145.1	119.7	2.7	33.6	23.1	58.7	18.3
	(0.1)	(0.2)	(110.3)	(17.5)	(12.0)	(0.3)	(3.1)	(1.5)	(1.2)	(1.1)

TABLE 2.5. Temperature and soil moisture averages of 3-year-old plots.
Compiled from Angel (2008).

	AVERAGE FROM 0-3 YEARS		
	BROWN	GRAY	MIXED
AVE. SOIL TEMP, -10 CM	15.7	15.1	16.5
SOIL MOISTURE, AVE. -50 CM	0.2	0.1	0.05
SOIL MOISTURE, AVE. - 200 CM	0.3	0.1	0.1

TABLE 2.6A. Infiltrated spoil water geochemistry for 3-year-plots, May 2008.

PLOT	alkalinity	F-	Cl-	SO ₄ ²⁻	NO ₃ -	Mg ²⁺	Si	Sr ²⁺	Al	Ca ²⁺	K ⁺	Na ⁺	Charge balance
	mg/L CaCO ₃						mg/L						
BROWN-1	119.3	0.18	2.93	262.45	0.19	40.53	3.41	0.19	0.01	69.00	4.82	19.08	-1.51
BROWN-2	307.3	0.28	4.12	797.11	4.77	147.89	2.05	0.42	0.04	204.36	13.48	23.66	2.51
GRAY-1	345.7	0.20	1.12	155.23	1.88	103.32	0.97	0.24	0.03	42.40	17.58	5.72	4.81
MIXED-1	268.9	0.23	1.58	210.36	51.63	79.50	1.30	0.29	0.02	68.69	12.16	8.31	7.55
MIXED-2	252.7	0.31	0.98	118.69	3.53	62.49	1.06	0.29	0.02	58.89	12.24	5.76	6.08

TABLE 2.6B. Field measurements for 3-year plots, May 2008.

	BROWN	GRAY	MIXED
TEMPERATURE, °C	20.2	19.1	19.0
EC, µS cm-1	830.5	830.0	1364.5
PH	8.027	8.307	8.166

TABLE 2.7. Summary of soil baiting isolates collected monthly from May to October 2007 and May 2008 for 3-year plots.

3-YEAR PLOTS	BROWN	GRAY	MIXED	FOREST SOILS
TOTAL ISOLATES TESTED BY PCR [†]	7	2	3	10
<i>Phytophthora cinnamomi</i>	0	0	0	8
<i>Phytophthora cryptogea</i>	1	0	0	0
<i>Mortierella</i> spp.	1	1	2	0
<i>Pythium</i> spp.	0	0	0	1
UNKNOWN	1	1	0	0

[†]Some isolates did not yield PCR products.

TABLE 2.8. Summary of soil baiting isolates collected monthly from May to October 2007 and May 2008 for 1-year plots.

1-YEAR PLOTS	BROWN	GRAY	MIXED	SHALE
TOTAL ISOLATES TESTED BY PCR [†]	6	1	2	0
<i>Phytophthora cinnamomi</i>	0	0	0	0
<i>Phytophthora cryptogea</i>	0	0	0	0
<i>Mortierella</i> spp.	2	0	1	0
UNKNOWN	1	0	1	0

[†]Some isolates did not yield PCR products.

TABLE 2.9. Summary of infiltrated water isolates from July 2007, Nov. 2007, and May 2008.

INFILTRATED PLOT WATERS-3 YEAR PLOTS	BROWN	GRAY	MIXED	CLEMENS FORK
TOTAL ISOLATES TESTED BY PCR [†]	7	0	0	4
<i>Phytophthora cinnamomi</i>	0	0	0	0
<i>Phytophthora cryptogea</i>	2	0	0	0
<i>Pythium</i> spp.	1	0	0	3
<i>Mortierella</i> spp.	1	0	0	0
UNKNOWN ZYGOMYCETE	0	0	0	1
UNKNOWN	3	0	0	0

[†]Some isolates did not yield PCR products.

TABLE 2.10. Characteristics of second-transfer isolates from soil baiting.

AGE OF PLOT	SAMPLE ID	COLONY MORPHOLOGY	MICROSCOPE (200X)	PCR IDENTIFICATION
MAY				
3-YEAR	BROWN-1	Appressed, poor growth	thin, branching hyphae; wiggly or straight, no swellings	*
	BROWN-1	Appressed, stellate	Many terminal swellings	*
1-YEAR	BEREA	Aerial, pettaloid, cottony	Cloudy under scope; swellings; gnarled appearance	<i>Phytophthora cinnamomi</i>
	BEREA	Aerial, pettaloid, cottony	Cloudy under scope; swellings	<i>Phytophthora cinnamomi</i>
	ROB	Aerial, cottony	Branching hyphae with swellings; coralloid	<i>Phytophthora cinnamomi</i>
JUNE				
3-YEAR	BROWN-2	flat colony; radial growth	Sporangium pear-shaped and some with papillate thickening	UNKNOWN FUNGI
	ROB	Aerial, cottony	Terminal and intaculary swellings; branching hyphae; gnarled appearance	<i>Phytophthora cinnamomi</i>
	BEREA	Aerial, cottony	Globose; zoospores inside	<i>Phytophthora cinnamomi</i>
JULY				
1-YEAR	MIXED-2	Flat colony, radial growth	wiggly hyphae with some swellings	UNKNOWN FUNGI
	ROB	Aerial, cottony	Thick, branching hyphae	*
AUGUST				
3-YEAR	BROWN-1	Fuzzy on top	Thin, straight branching hyphae	<i>Mortierella</i> sp.
	GRAY-1	Fuzzy on top, some aerial growth	Thin straight branching hyphae	UNKNOWN
	BROWN-1	Splotchy surface growth	Straight, branching, mostly on surface of agar with swellings	UNKNOWN
1-YEAR	MIXED-1	Fuzzy on top; opaque growth	Thin, straight branching hyphae	ZYGOMYCETE

	ROB	Aerial, cottony	Straight, branching, with swellings	<i>Phytophthora cinnamomi</i>
			SEPTEMBER	
1-YEAR	BROWN-1	Flat, radial colony	Sparse growth, occasional swellings	*
	ROB	Appressed growth throughout plate	Straight, branching hyphae	*
			OCTOBER	
	BROWN-1	Fuzzy, all of plate, slightly aerial	Long, straight branching hyphae, a few round terminal swellings	*
3-YEAR	MIXED-2	Surface snowflake-like growth, on center of plate, appressed	Intaculary swellings, branching complex hyphae	*
	BROWN-2	Branching and fuzzy; covers half of plate	Branching hyphae with intaculary swellings	<i>Mortierella</i> sp.
1-YEAR	GRAY-1	All of surface covered--snowflake-like growth	Intaculary swellings; branching, complex hyphae on surface only	*
	ROB	Aerial, cottony	A few liminiform; terminal, globose papillate swellings	<i>Pythium</i> sp.
			MAY 2008	
	BROWN-2	Colony covers surface and into agar medium; radial habit	Branching, coralloid mycelium with occasional swellings	<i>Phytophthora cryptogea</i>
3-YEAR	MIXED-1	Radial colony growing on agar surface	Sparse growth, occasional small intaculary swellings	<i>Mortierella</i> sp.
	MIXED-2	Radial colony growing on agar surface	Sparse growth, occasional small intaculary swellings	<i>Mortierella</i> sp.
	GRAY-1	Radial colony growing on agar surface	Sparse growth, occasional small intaculary swellings	<i>Mortierella</i> sp.
1-YEAR	BROWN-1	Nickel sized colony, on surface	Sparse growth, occasional small intaculary swellings	<i>Mortierella</i> sp.
	ROB	Slight aerial growth, radiates outward, nearly covers plate	Straight, branching hyphae	*

* indicates that DNA extracts were not amplified during PCR reactions

† indicates DNA sequencing resulted in poor sequences

TABLE 2.11. Characteristics of second-transfer isolates from water filtering.

SAMPLE ID	COLONY MORPHOLOGY	MICROSCOPE (200X)	PCR IDENTIFICATION
JULY 2007			
BROWN-1	Poor growth on surface of agar	Globose, papillate terminal swellings	*
BROWN-1	Poor growth on surface of agar	Straight hyphae with globose clusters attached	*
CLEMENS	Cottony growth throughout medium	Semi-straight, branching hyphae; some swellings	UNKNOWN ZYGOMYCETE
NOVEMBER 2007			
BROWN-1-2	Radial colony growing on agar surface	Sparse growth, occasional small intaculary swellings	<i>Mortierella</i> sp.
BROWN-2-2	Appressed, poor growth	Mostly straight hyphae with terminal swellings	†
BROWN-2-5	Growth on center of plate, appressed	Mostly straight hyphae with terminal swellings	<i>Pythium</i> sp.
MAY 2008			
ROB WATER 1	Aerial, cottony	Straight, branching hyphae with various sized swellings:terminal and intaculary	<i>Pythium undulatum</i>
ROB WATER 2	Aerial, cottony	“	<i>Pythium undulatum</i>
ROB WATER 3	Aerial, cottony	“	<i>Pythium undulatum</i>
BROWN-1-T2	Fairly cottony colonies	Anastomizing hyphae,occasional swellings	<i>Phytophthora cryptogea</i>
BROWN-1-T3	Fairly cottony colonies	“	<i>Phytophthora cryptogea</i>

* indicates that DNA extracts were not amplified during PCR reactions

† indicates DNA sequencing resulted in poor sequences



FIGURE 2.1. Aerial view of the 2005 loose-dumped plots at the Bent Mountain surface mine in Pike County, Kentucky. The size of each plot is approximately 0.4-hectare.



FIGURE 2.2. Aerial view of the 2007 loose-dumped plots. The size of each plot is approximately 0.2-hectare.

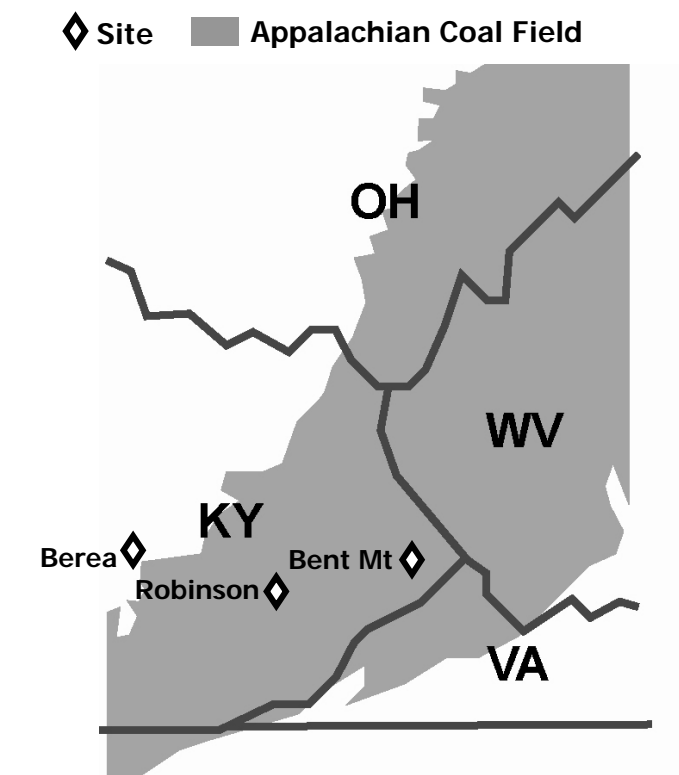


FIGURE 2.3. Location of study sites.



FIGURE 2.4. Baiting for *Phytophthora* spp. in soil.

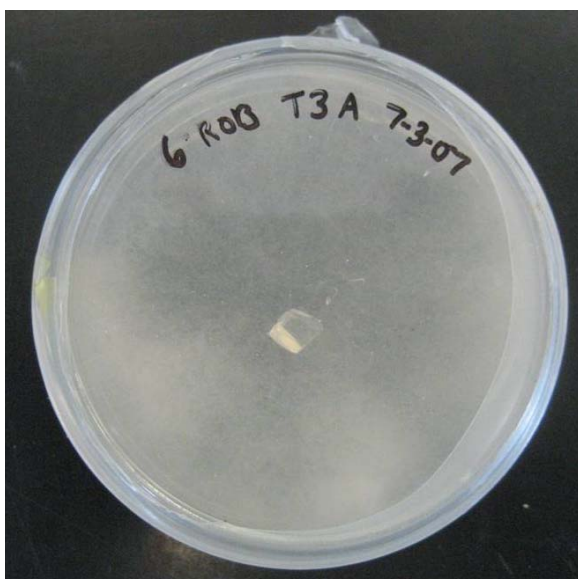


FIGURE 2.5. Culture of *Phytophthora cinnamomi*.

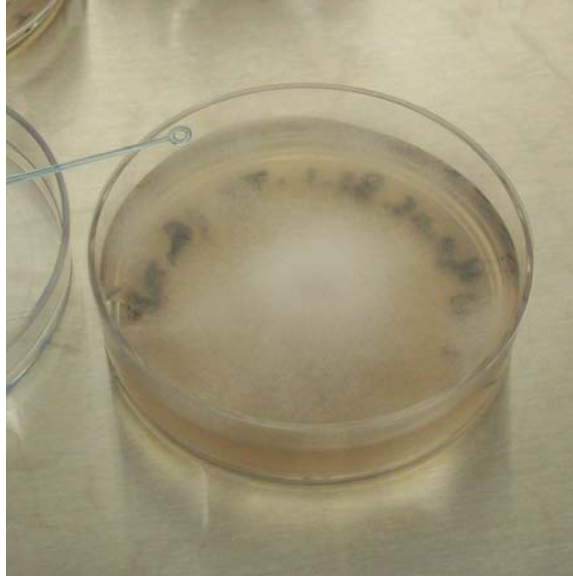


FIGURE 2.6. Culture of *Phytophthora cryptogea* on PARPH-V8 agar.

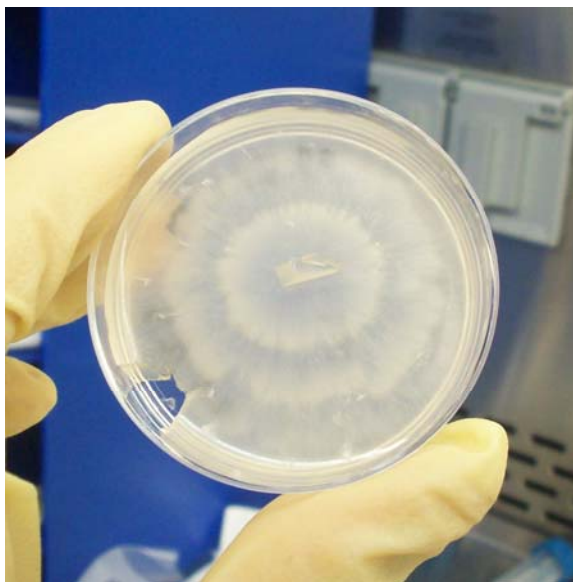


FIGURE 2.7. Culture of *Mortierella*.

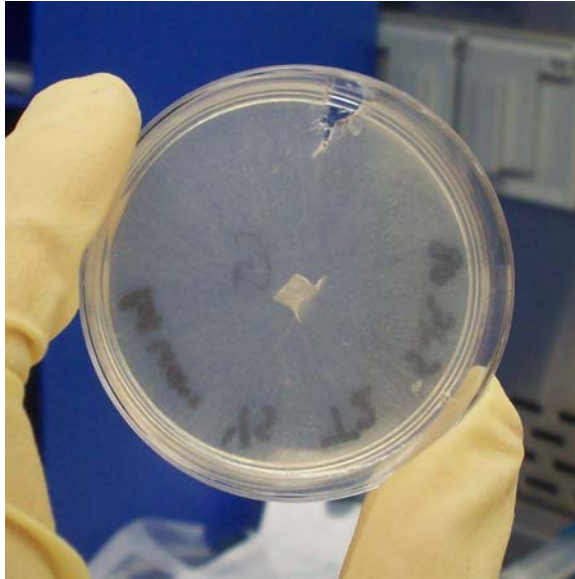


FIGURE 2.8. Culture of *Pythium undulatum*.

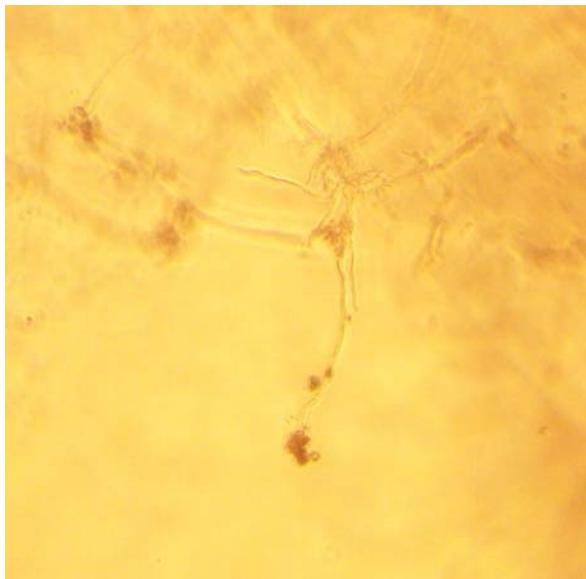
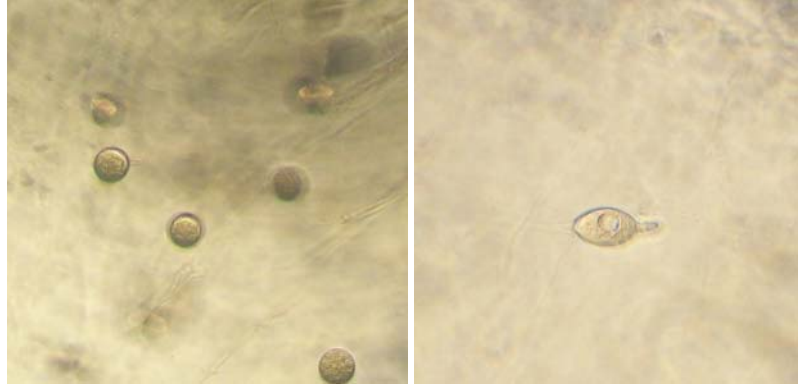


FIGURE 2.9. 200x of *Phytophthora cryptogea*.



FIGURES 2.10 AND 2.11. Agar culture of *Pythium undulatum* at 200x.

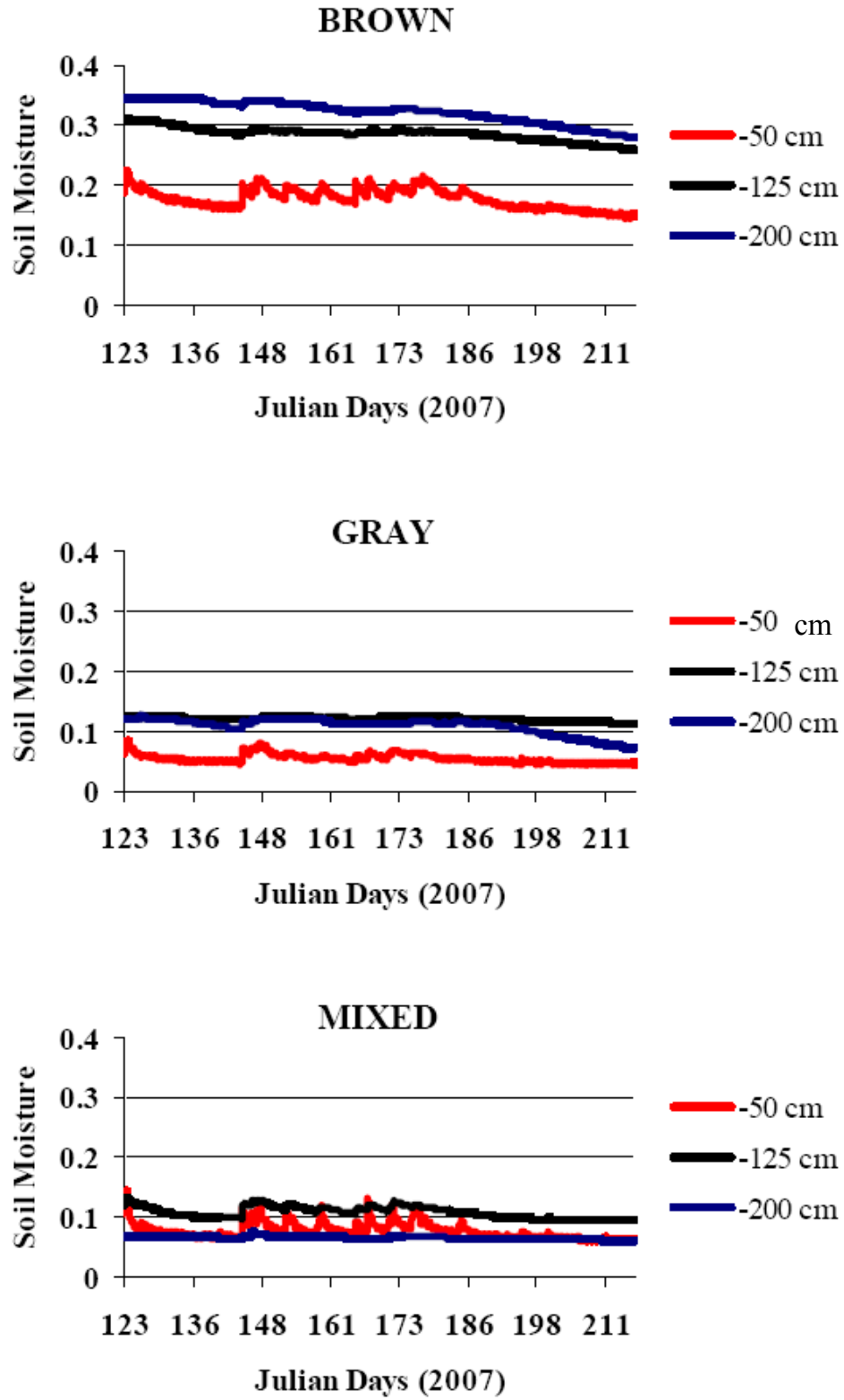


FIGURE 2.12 Soil moisture as measured by moisture content probes from 1 May 2007 to 5 August 2007 at -50, -125, and -200 cm. From Angel (2008).

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GGAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC 60
CTAAAAAACTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGGCCTGCTCTGGGCGG 120
CGGCTGTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC 180
GTTTGGGTCCCTCTCGGGGGAAGTGAAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT 240
ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATTAGCAACTTT5.8 SCAGCAGT 300
GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT 360
GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG 420
GAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGGCTCTCTTCCTTCCGTGTAGTCGG 480
TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC 540
CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG 600
AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT 660
GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG 720
CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT 800
GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTGAGGGTCGATCCATTTGGGAACTCTG 860
TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATTATCAGGCA 920
AGATTACCCGCTGAACTTAAGCATATCAATAAGCGGA 957

```

Fig. 2.13. Consensus sequence of the intergenic spacer regions (ITS) of the *P. cinnamomi* isolates from Berea and Robinson forests showing partial sequence of the ribosomal genes 18S and 28S genes and the complete sequences of the 5.8 gene and of ITS-1 and ITS-2. Primer annealing regions are indicated by red arrows and letters; solid arrows indicate the forward and reverse primers prITS-6 and prITS-4, and dashed arrows indicate forward and reverse primers prCIN3A and prCINTS4. Alignments were performed using CLUSTALW in ChromasPro version 1.33.

BS1_05_L -----GCCCCAC
BS1_05_K -----ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
P. cryptogea GAAGTAAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
BS2_05_A TGAAGGGGAATGCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
*** **

BS1_05_L CTAAAAAACTTTCCACGTGAACCGTATCAACCTTTTTTAAATTGGGGGCTTCCGTCTGGCC
BS1_05_K CTAAAAAACTTTCCACGTGAACCGTATCAACCTTTTTTAAATTGGGGGCTTCCGTCTGGCC
P. cryptogea CTAAAAAACTTTCCACGTGAACCGTATCAACCTTTTTTAAATTGGGGGCTTCCGTCTGGCC
BS2_05_A CTAAAAAACTTTCCACGTGAACCGTATCAACCTTTTTTAAATTGGGGGCTTCCGTCTGGCC

BS1_05_L GGCCGGTTCTCGGCTGGCTGGGTGGCGGCTCTATCATGGCGACCGCTTGGGCCTCGGCCT
BS1_05_K GGCCGGTTCTCGGCTGGCTGGGTGGCGGCTCTATCATGGCGACCGCTTGGGCCTCGGCCT
P. cryptogea GGCCGGTTCTCGGCTGGCTGGGTGGCGGCTCTATCATGGCGACCGCTTGGGCCTCGGCCT
BS2_05_A GGCCGGTTCTCGGCTGGCTGGGTGGCGGCTCTATCATGGCGACCGCTTGGGCCTCGGCCT

BS1_05_L GGGCTAGTAGCGTATTTTTTAAACCCATTCTAATTACTGAATATACTGTGGGGACGAAA
BS1_05_K GGGCTAGTAGCGTATTTTTTAAACCCATTCTAATTACTGAATATACTGTGGGGACGAAA
P. cryptogea GGGCTAGTAGCGTATTTTTTAAACCCATTCTAATTACTGAATATACTGTGGGGACGAAA
BS2_05_A GGGCTAGTAGCGTATTTTTTAAACCCATTCTAATTGAAAATAAAGTGTGGGAAGAAA
***** * ***** *

BS1_05_L GTCTCTGCTTTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGAT
BS1_05_K GTCTCTGCTTTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGAT
P. cryptogea GTCTCTGCTTTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGAT
BS2_05_A GTCTCTGCTTTTAACTAGATAGCAACCATCAGCAGTGGATGTCTAGGCTCGCACATCGAT

BS1_05_L GAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCAGTGAGTCATCGAAA
BS1_05_K GAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCAGTGAGTCATCGAAA
P. cryptogea GAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCAGTGAGTCATCGAAA
BS2_05_A GAAGAACGTTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCAGTGAGTCATCGAAA

BS1_05_L TTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCTGTATCAGTGTCCGT
BS1_05_K TTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCTGTATCAGTGTCCGT
P. cryptogea TTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCTGTATCAGTGTCCGT
BS2_05_A TTTTGACCCCATATTGCACTTCCGGGTTAGTCCTGGGAGTAGACCTGTTTCAGTGTCCGT
***** * *****

BS1_05_L AACTAACTTGGCTCCCTTCCTTCCGTGTAGTCGGTGGATGGGGACGCGCAGATGTGAA
BS1_05_K AACTAACTTGGCTCCCTTCCTTCCGTGTAGTCGGTGGATGGGGACGCGCAGATGTGAA
P. cryptogea AACTAACTTGGCTCCCTTCCTTCCGTGTAGTCGGTGGATGGGGACGCGCAGATGTGAA
BS2_05_A ACAGTAACTTGGCTCCCTTCCTTCCGTGTAGTCGGTGGATGGGGACGCGCAGATGTGAA
*** *****

BS1_05_L GTGTCTTGCGGCTGGTCTTCGGTCCGGCTGCGAGTCCTTTGAAATGTACTAACTGTACT
BS1_05_K GTGTCTTGCGGCTGGTCTTCGGTCCGGCTGCGAGTCCTTTGAAATGTACTAACTGTACT
P. cryptogea GTGTCTTGCGGCTGGTCTTCGGTCCGGCTGCGAGTCCTTTGAAATGTACTAACTGTACT
BS2_05_A GTGTCTTGCGGCTGGTAATCGGTCCGGCTGCGAGTCCTTTGAAATGTAGTAACTGTACT
***** *****

BS1_05_L TCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGGAGGCTGCCTGTGTGGCATGTCCG
BS1_05_K TCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGGAGGCTGCCTGTGTGGCATGTCCG
P. cryptogea TCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGGAGGCTGCCTGTGTGGCATGTCCG
BS2_05_A TCTCTTTGCTCGAAAAGCGTGACGTTGGTGGTTGTGGAGGCAGCCTGTGTGGCATGTCCG
***** *****

BS1_05_L CGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGTGTTTCGATTTCGCGGTATGATCGGC
BS1_05_K CGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGTGTTTCGATTTCGCGGTATGATCGGC
P. cryptogea CGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGTGTTTCGATTTCGCGGTATGATNGGC
BS2_05_A CGACCGGTTTCATCTGCTGCGGCGTTTAATGGAGGAGTGTTTCGATTTCGCGGTATGATCGGC
***** *****

BS1_05_L TTCGGCTGAACAGACGCTTATTGGGTGCTTTTCCTGCTGTGGCTGGATGGACTGGTGAAC
BS1_05_K TTCGGCTGAACAGACGCTTATTGGGTGCTTTTCCTGCTGTGGCTGGATGGACTGGTGAAC
P. cryptogea TTCGGCTGAACAGACGCTTATTGGGTGCTTTTCCTGCTGTGGCTGGATGGACTGGTGAAC
BS2_05_A TTCGGCTCAACAGGAACCTACGGGGTGCTTTTCATGCAGGGGCAGGATGGACTGGTTAAC
***** *****

BS1_05_L	CGTAGCTGTGCTAGGCTTTGGCTTTTGAACCGGTGGTGTGGTGCGAAGTAGGGTGTCTGTT
BS1_05_K	CGTAGCTGTGCTAGGCTTTGGCTTTTGAACCGGTGGTGTGGTGCGAAGTAGGGTGTCTGTT
<i>P. cryptogea</i>	CGTAGCTGTGCTAGGCTTTGGCGTTTGAACCGGCGGTGTGGTGCGAAGTAGGGTGTCTGTT
BS2_05_A	AGTAGTTGTGCTAGGCTTTGGCGTTTGAACCGGTGGTGTGGTGAGAAGTAGGGTGTCTGTT

BS1_05_L	CCGGCGCAAGCTGGGGTGGACGAGGGTCGATCCATTTGGGAAACGTT-GTGTGCGCTTCG
BS1_05_K	CCGGCGCAAGCTGGGGTGGACGAGGGTCGATCCATTTGGGAAACGTT-GTGTGCGCTTCG
<i>P. cryptogea</i>	CCGGCGCAAGCTGGGGTGGACGAGGGTCGATCCATTTGGGAAACGTT-GTGTGCGCTTCG
BS2_05_A	CCGGCGCAAGAGGGGGTGGACGAGGGTCGATCCACTTGGGAAACGAGAGTGTGCGCTTCG

BS1_05_L	GCGCGC-ATCTCAA-TTGGACCTGATATCAGCAAGATTACCCGCTGAACCTTAAGCATATC
BS1_05_K	GCGCGC-ATCTCAA-TTGGACCT-----
<i>P. cryptogea</i>	GCGCGC-ATCTCAA-TTGGACCT-----
BS2_05_A	GCGCGCTATCTCAAATTGGGATCCCCG-----

BS1_05_L	ATAAAGCGGAGGAAA
BS1_05_K	-----
<i>P. cryptogea</i>	-----
BS2_05_A	-----

Figure 2.14. CLUSTAL W (1.83) alignment of ITS sequences of *Phytophthora cryptogea* (Accession Number EU 000138.1) and ITS sequences of PCR amplification products using primers ITS 4 and ITS 6 and total DNA of *Phytophthora* colonies recovered from brown sandstone spoil (BS2_05_A) and infiltrated brown sandstone spoil water. Colonies were recovered from brown sandstone spoil by leaf baiting from spoil immersed in water using rhododendron leaves, and from filters used for filtering samples of infiltrated spoil water. Leaf disks and filters were placed in plates of PARPH-V8 selective medium for *Phytophthora*.

CIN_Berea_s4	-----
CIN_RF_s18	-----
CIN_RF_s9	-----
<i>P. cinnamomi</i>	-----AGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
RF_ITS_s6	-GAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
RF_ITS_s18	-----CCACAC
Berea_ITS_s10	-GAAGGTGAAGTCGTAACAAGGTT-CCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
RF_ITS_s9	-GAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
Berea_ITS_s4	GGAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC
Berea_ITS_s5	GGAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCACAC

CIN_Berea_s4	-----TCATTAGTTGGGGCCTGCTCTGGGCGG
CIN_RF_s18	-----TCCATATTAGTTGGGGCCTGCTCTGGGCGG
CIN_RF_s9	-----TCTGGGCGG
<i>P. cinnamomi</i>	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
RF_ITS_s6	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
RF_ITS_s18	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
Berea_ITS_s10	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
RF_ITS_s9	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
Berea_ITS_s4	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG
Berea_ITS_s5	CTAAAAAAGTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGCCTGCTCTGGGCGG

CIN_Berea_s4	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
CIN_RF_s18	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
CIN_RF_s9	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
<i>P. cinnamomi</i>	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
RF_ITS_s6	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
RF_ITS_s18	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
Berea_ITS_s10	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
RF_ITS_s9	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
Berea_ITS_s4	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC
Berea_ITS_s5	CGGCTGTTCGATGTCAAAGTCGACGGCTGCTGCTGCGTGGCGGGCCCTATCACTGGCGAGC

CIN_Berea_s4	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
CIN_RF_s18	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
CIN_RF_s9	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
P. cinnamomi	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
RF_ITS_s6	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
RF_ITS_s18	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
Berea_ITS_s10	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
RF_ITS_s9	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
Berea_ITS_s4	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT
Berea_ITS_s5	GTTTGGGTCCCTCTCGGGGGAAGTCTGAGCTAGTAGCCTCTCTTTTAAACCCATTCTGTAAT

CIN_Berea_s4	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
CIN_RF_s18	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
CIN_RF_s9	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
P. cinnamomi	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
RF_ITS_s6	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
RF_ITS_s18	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
Berea_ITS_s10	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
RF_ITS_s9	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
Berea_ITS_s4	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT
Berea_ITS_s5	ACTGAACATACTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTTCAGCAGT

CIN_Berea_s4	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
CIN_RF_s18	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
CIN_RF_s9	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
P. cinnamomi	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
RF_ITS_s6	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
RF_ITS_s18	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
Berea_ITS_s10	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
RF_ITS_s9	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
Berea_ITS_s4	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT
Berea_ITS_s5	GGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATT

CIN_Berea_s4	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
CIN_RF_s18	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
CIN_RF_s9	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
<i>P. cinnamomi</i>	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
RF_ITS_s6	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
RF_ITS_s18	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
Berea_ITS_s10	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
RF_ITS_s9	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
Berea_ITS_s4	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG
Berea_ITS_s5	GCAGGATTCACTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGG

CIN_Berea_s4	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
CIN_RF_s18	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
CIN_RF_s9	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
<i>P. cinnamomi</i>	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
RF_ITS_s6	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
RF_ITS_s18	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
Berea_ITS_s10	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
RF_ITS_s9	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
Berea_ITS_s4	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG
Berea_ITS_s5	GAGTATGCCTGTATCAGTGTCCGTACATCAAACCTTGGCTCTCTTCCTTCCGTGTAGTCGG

CIN_Berea_s4	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
CIN_RF_s18	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
CIN_RF_s9	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
<i>P. cinnamomi</i>	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
RF_ITS_s6	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
RF_ITS_s18	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
Berea_ITS_s10	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
RF_ITS_s9	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
Berea_ITS_s4	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC
Berea_ITS_s5	TGGATGGAGGTGCCAGACGTGAGGTGTCTTGCGGGCGGTCTTCGGACTGGCTGTGAGTCC

CIN_Berea_s4	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
CIN_RF_s18	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
CIN_RF_s9	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
<i>P. cinnamomi</i>	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
RF_ITS_s6	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
RF_ITS_s18	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
Berea_ITS_s10	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
RF_ITS_s9	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
Berea_ITS_s4	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG
Berea_ITS_s5	CTTGAAATGTACTGAACTGTACTTCTCTTTGCTCGAAAAGCGTGACGTTGCTGGTTGTGG

CIN_Berea_s4	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
CIN_RF_s18	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
CIN_RF_s9	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
<i>P. cinnamomi</i>	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
RF_ITS_s6	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
RF_ITS_s18	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
Berea_ITS_s10	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
RF_ITS_s9	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
Berea_ITS_s4	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT
Berea_ITS_s5	AGGCTGCCTGTATGGCCAGTCGGCGACCGGTTTGTCTGCTGCGGCGTTTAATGGAGGAGT

CIN_Berea_s4	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
CIN_RF_s18	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
CIN_RF_s9	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
<i>P. cinnamomi</i>	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
RF_ITS_s6	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
RF_ITS_s18	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
Berea_ITS_s10	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
RF_ITS_s9	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
Berea_ITS_s4	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG
Berea_ITS_s5	GTTTCGATTTCGCGGTATGGTTGGCTTCGGCTGAACAAAGCGCTTATTGGATGTTCTTCCTG

CIN_Berea_s4	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
CIN_RF_s18	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
CIN_RF_s9	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
<i>P. cinnamomi</i>	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
RF_ITS_s6	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
RF_ITS_s18	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
Berea_ITS_s10	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
RF_ITS_s9	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
Berea_ITS_s4	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT
Berea_ITS_s5	CTGTGGCGGTACGGATCGGTGAACCGTAGCTGTGCTAGGCTTGGCGTTTGAACCGGCGGT

CIN_Berea_s4	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
CIN_RF_s18	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
CIN_RF_s9	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
<i>P. cinnamomi</i>	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
RF_ITS_s6	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
RF_ITS_s18	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
Berea_ITS_s10	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
RF_ITS_s9	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
Berea_ITS_s4	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG
Berea_ITS_s5	GTTGTTGCGAAGTAGGGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAACCTCTG

CIN_Berea_s4	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCAA-----
CIN_RF_s18	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCAA-----
CIN_RF_s9	TGTCTCTGCGGCGCACTTG-----
<i>P. cinnamomi</i>	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA
RF_ITS_s6	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA
RF_ITS_s18	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAA-----
Berea_ITS_s10	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA
RF_ITS_s9	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA
Berea_ITS_s4	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA
Berea_ITS_s5	TGTCTCTGCGGCGCACTTGTGTGCTTGTGGTGGCATCTCAATTGGACCTGATATCAGGCA

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CIN_Berea_s4      -----
CIN_RF_s18        -----
CIN_RF_s9         -----
P. cinnamomi    AGATTACCCGCTGAACTTAAGCAAC-----
RF_ITS_s6         AGATTACCCGCTGAACTTAAGCATATCAATAAGCGGACCTGATATCAGGCA
RF_ITS_s18        -----
Berea_ITS_s10     AAGTT-----
RF_ITS_s9         AGATTACCCGCTGAACTTAAGCATACAAAAAACGGGGAGA-----
Berea_ITS_s4      AGATTACCCGCTGAACTTAAGCA-----
Berea_ITS_s5      AGATTACCCGCTGAACTTAAGCATATCA-----

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Figure 2.15. CLUSTAL W (1.83) multiple sequence alignment of sequences obtained by PCR amplification of the ITS region from total DNA extracted from colonies recovered on PARPH-V8 from forest soils by leaf baiting. The sequences designated RF_ITS_s6, RF_ITS_s9, RF_ITS_s18, Berea_ITS_s4, Berea_ITS_s5, Berea_ITS_s10, were obtained by amplification of total DNA using primers ITS4 and ITS6. Samples from Robinson Forest were RF_s6, RF_s9, RF_s18, and samples Berea_s4, Berea_s5, Berea_s10 were collected from Berea College Forest. The sequences designated CIN_Berea_s4, CIN_RF_s9, CIN_RF_18 were obtained using primers CIN3A and CINITS 4 for amplification of DNA from samples Berea s4, RF s9 and RF s18. The sequence of *Phytophthora cinnamomi* GenBank Accession number AY302147 was used in the alignments.

APPENDIX A. PAR(PH)-V8 SELECTIVE MEDIUM FOR *PHYTOPHTHORA* SPECIES

References: Jeffers and Martin, 1986; Ferguson and Jeffers, 1999.

COMPONENTS OF PAR(PH)-V8 MEDIUM

	<i>To be added in prepared stock solutions</i>
100 mL V8 juice	0.0667 g Terraclor (75% PCNB)
1 g CaCO ₃	0.05 g Hymexazole
950 mL distilled water	0.01 g Rifamycin-SV (sodium salt)
15 g Difco Bacto Agar	0.01 g Delvocid (50% pimaricin)
	0.25 g sodium ampicillin

CLARIFIED V8 JUICE

Buffer V8 by adding 1 g CaCO₃ per 100 mL V8, or 20.4 g per 1L can. Stir 30 minutes with a magnetic stirrer. Centrifuge at 7000 rpm for 10 minutes. Decant and collect upper phase.

STOCK SOLUTIONS

MATERIAL	AMOUNT	WATER	USE PER 1 LITER
TERRRACLOR (75% PCNB)	0.56 g	25 ml	3.0 ml
HYMEXAZOLE	0.50 g	25 ml	2.5 ml
RIFAMYCIN-SV (SODIUM SALT)	0.25 g	25 ml	1.0 ml
DELVOCID (50% PIMARCIN)	0.25 g	25 ml	1.0 ml
SODIUM AMPICILLIN	0.25 g	DRY	0.25 g

PREPARATION OF 1 LITER OF PAR(PH)-V8 MEDIUM

1. Add the following to a 2 L flask and mix with a large magnetic stir bar: 50 ml clarified V8 concentrate, 950 ml distilled H₂O, and 15 g Difco Bacto Agar.
2. Autoclave for 20 min at 121°C and 15 p.s.i., then put in a waterbath at 50°C to cool.
3. After the medium cools to 50°C, add each of the stock solutions under the hood.
4. Stir well and pour plates under the hood. Allow plates to cool with the lid partially off.
5. Store plates inverted in a plastic bag in the dark in a refrigerator. Plates generally retain antibiotic properties for six months.

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